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FLIGHT ACTIVITY IN THE BLOWFLY *CALLIPHORA ERYTHROCEPHALA*, IN RELATION TO LIGHT AND RADIANT HEAT, WITH SPECIAL REFERENCE TO ADAPTATION

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1. INTRODUCTION

Many insects depend for survival on wide dispersal by flight. Small insects are carried about passively by air currents (Hardy & Milne, 1937, 1938; Freeman, 1945; Gislén, 1948; Johnson, 1954) and the displacement of even such a large and powerful form as a locust depends largely on the wind direction during flight (Waloff, 1946; Rainey & Waloff, 1948). Spontaneous flight activity is on the whole most obvious in fine sunny weather, but we have little information on the way in which various weather factors might affect it. The subject is difficult to study in the field because most of the factors commonly change together.

Laboratory studies by Davies (1935, 1936) and Broadbent (1949) have concerned the effect of temperature, humidity, wind speed and light intensity on aphids, and Nicholson (1934) has described the effect of temperature on the flight activity of various species of blowflies. There appears to be, however, no detailed account of the way in which light and radiant heat may operate at intensities comparable to those found in sunshine, and little attention has so far been given to the effects of adaptation. The time course of adaptation is of considerable ecological significance, for the conditions to which insects are exposed in sunny weather consists of a continuous series of changes, arising both from changes in the weather and from movements of the insects themselves. The change in activity in response to any particular change of conditions is therefore closely dependent on the rate and extent of adaptation. The experiments described in this paper were carried out to clarify these matters.

The blowfly, *Calliphora erythrocephala* Mg., proved a good subject for these experiments. Observations were made upon a small population of these insects confined in a transparent chamber in a small wind tunnel, and subjected to changes in the experimental factors over a range of conditions comparable to those normally encountered in the field.

2. METHODS

General methods

The blowflies used in this study were bred in muslin cages, and the more active individuals were selected for experiment by taking those which flew actively about the cage and came to rest on the walls nearest the light. They were used at an age of between 2 weeks and 1 or 2 months, according to the season, and males and females were used together in roughly equal numbers. Flight activity was observed by confining the insects in an oblong celluloid chamber with copper or nylon gauze ends (Fig. 1). Internal measurements were $21{\cdot}5 \times 15{\cdot}5 \times 6{\cdot}5$ cm, and when fitted into the small wind tunnel described previously (Digby, 1955), a current of air could be blown through to keep the temperature relatively constant when using radiation strengths sufficient to produce heating. All observations of activity were visual, the insect chamber having behind and below it a piece of white card against which the insects were silhouetted in such a way that their movements could be followed with ease. Insects were commonly used as groups of forty, the total number of flights seen in 45 sec. (or, when very active, in 15 sec.) being recorded once per minute and reduced to flights per specimen per minute. Little difficulty was experienced in counting their flights up to a rate of about 160/min., an average rate of four flights per specimen per minute. Above this rate the occasional coincidence of several flights necessitated a certain amount of estimation, which is, however, unlikely to lead to errors of observation of more than 5 % at a rate of 200 flights/min. (5 flights/specimen/min.). Where necessary, groups of twenty insects were used for confirmatory experiments. A given batch of insects was commonly run for up to 5 hr. unless the members showed signs of excessive fatigue by an unduly rapid fall in flight activity and excitability. After each run the insects were returned to the cages for use later on.

All the experiments were carried out at a constant wind speed of 0·5 m./sec. as measured by a hot wire anemometer and Pitot tube close to the centre of the wind channel immediately down-wind from the insect chamber. As the insects were distributed and moved about the chamber more or less at random, those flying through the centre were subjected to wind of the measured velocity, while the others at rest on the sides would experience lower speeds.

The air in the tunnel was usually in free circulation with that of the laboratory, temperature being raised as necessary by heating elements in the tunnel. Most of the experiments were carried out over a period of time, the temperature and humidity, measured by wet- and dry-bulb thermometers, ranging from 17·0 to 25·8° C. and from 45 to 73 % R.H. between experiments. In any one experiment at or below light intensities of 2600 lux the temperature was held constant to 1·0° C. and humidity remained steady apart from changes resulting from changes of temperature. Over the range used, variation of temperature and humidity between experiments has negligible influence on the magnitude of the reaction to light, and the results are therefore considered as one group.

Temperature profiles within the insect chamber were measured by a fine copper-constantan thermocouple used with a unipivot galvanometer type LX.

Light and radiant heat

Light in the range from 20 to 41,000 lux was supplied by tungsten gas-filled bulbs mounted vertically overhead. To keep the colour approximately constant, light intensity was adjusted by using one of a series of bulbs of various strengths (2 kW., 150 W. and 15 W.), by moving the particular bulb up or down, and by inserting various thicknesses of wire gauze or paper between it and the insect chamber. The 2 kW. bulb was under-run to reduce its colour temperature to approximately that of the others and to lengthen its life. Intensities below 20 lux were obtained by using a small 3·5 V. bulb with an opal and various neutral filters. The observer wore heavily smoked glasses during the initial periods at high light intensity and removed them when the light was reduced, to ensure a sufficient degree of dark-adaptation to be able to see the insects during the period at low intensity.

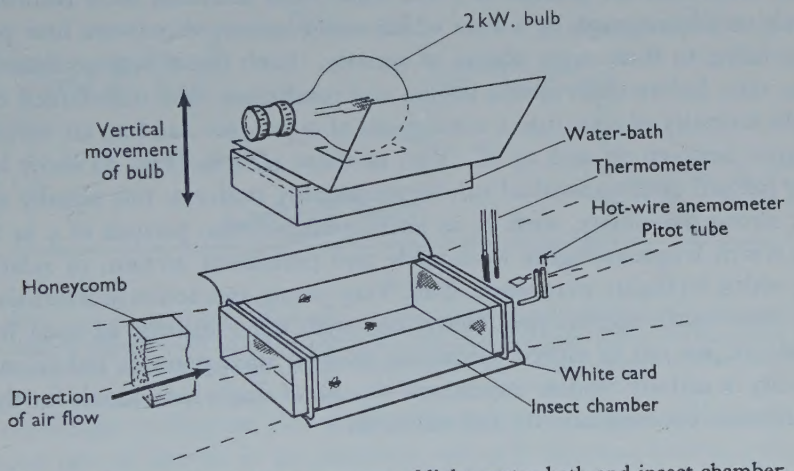

Fig. 1. Diagram showing arrangement of light, water-bath and insect chamber.

Light intensity was measured at the centre of the insect chamber by a Weston photographic exposure meter covered by a diffusing opal or by a selenium photocell with an opal and a Cambridge unipivot galvanometer, the instruments being calibrated against a 1000 W. standard photometric lamp at 2854° K. Radiant heat was measured by a small pyrheliometer (Digby, 1955).

The 2 kW. bulb was used with a 3·5 cm. depth of water in a Perspex trough as a heat filter, increasing the efficiency of the radiation to approximately 34 lumens per watt. This is below that of daylight when there is little or no cloud, which ranges between roughly 80 and 125 lumens per watt (Atkins & Poole, 1936; Blackwell, 1954 and personal communication). Values of illumination at and above 2600 lux in these experiments therefore cause roughly three times as much heating as do the corresponding illumination intensities of daylight. Heating by the other bulbs used

without the water-bath is roughly twice as great for any given light intensity, but at the strengths used this was unimportant.

Conditions were varied over a range of values of the same order as those found in the field. Illumination ranged up to 87,000 lux, while the maximum for daylight is above 130,000 lux in summer with brightly lit cloud in the sky. Radiation intensity ranged up to 3·2 cal./cm^2./min., while the maximum radiation for daylight is a little above 1·5 cal./cm.^{2}/min. or possibly about 2·0 cal./cm.^{2}/min. when all sources are considered. Air and surface temperatures in the experiments were comparable to those found under the conditions prevailing close to the ground in summer.

3. ACTIVITY IN THE INDIVIDUAL AND IN THE POPULATION

Activity in the individual

In any individual the activity shown in the insect chamber at any one moment may be referred to one of four phases: quiescence, grooming, walking and flight. In one set of experiments individual insects confined in the chamber were watched under constant conditions for periods of 1 hr., while their activities were recorded continuously on a kymograph by a lever which could be moved between four positions corresponding to these main phases of activity. Each insect was acclimatized for 10 to 20 min. before observations began, and conditions were maintained constant at a light intensity of 2600 lux, a wind speed of 0·5 m./sec., and an air temperature at a figure between 22 and 25° C. Two of these records (Fig. 2) show how the activity pattern of an individual can be remarkably uniform, but equally well can exhibit strong periodicity, such as an alternation between periods of 5 or 10 min. during which frequent flights were made and periods of 20 min. of relative rest during which no flights were made at all. Very young and active individuals do not at first show such marked periodicity, but with older insects, as used for most experiments, six out of eleven specimens showed such periodic behaviour. This periodicity in activity renders unpractical the use of single individuals for the study of the relation between activity and stimulus.

Activity in the group

If, however, a sufficiently large group of individuals is studied as a whole, the average activity becomes sufficiently smoothed to permit the average relation of activity to stimulus to be followed with some degree of confidence. Fig. 3 shows how the flight activity of a group of forty flies decreases with reduction of light intensity, the insects showing marked photokinesis; 'high photokinesis' in the terminology of Fraenkel & Gunn (1940).

It is desirable to observe as large a group of insects as possible in order to reduce the irregularities arising from individual behaviour, but owing to the limitations of visual recording a group of forty flies proved most convenient.

Mutual stimulation

In considering such changes in the average activity of a group it is necessary to investigate possible interaction between individuals. In the breeding cages under

quiet, warm conditions and low illumination there is often an alternation in activity of the whole population, periods of very active flight alternating with periods of almost total quiescence. Such obvious periodicity in the behaviour of the group as a whole did not occur in the insect chamber under the conditions described, suggesting mutual stimulation to be less than in the breeding cages.

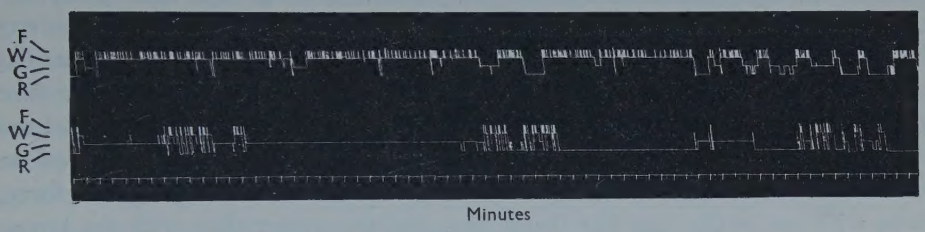

Fig. 2. Activity of two individual specimens of *Calliphora* in the insect chamber, showing continuous and rhythmic activity respectively. F, flights; W, walking; G, grooming; R, at rest. Time marker in minutes.

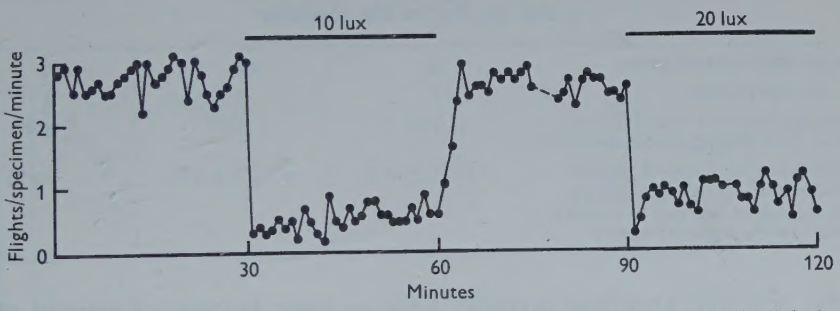

Fig. 3. Activity of a group of forty specimens, in a typical experiment during which the light intensity was changed from 2600 to 10 lux and back, and to 20 lux and back. The curve shows the decrease of activity with decrease of light intensity followed by a certain amount of recovery.

It is reasonable to suppose that such interaction, if it occurs, will be a function of the total rate of activity in the chamber, and will therefore lead to a change in the average rate of flight activity when the number of insects in the chamber is varied, other factors remaining constant.

Two series of experiments were therefore carried out to investigate the effects of crowding. Both were carried out at 2600 lux and at 17·0° or 22·0° C. maintained constant to 0·25° C. by means of a heating element in the air stream.

In the first series the activity of a single marked individual was investigated in relation to the presence of four similar unmarked individuals. In half the experiments one marked and four unmarked flies were confined in the chamber for 20 min. to become acclimatized, and then for a further 40 min. during which the flights were recorded. Half-way through the period of observation the unmarked members were removed quietly by a suction tube allowing comparison of the activity of the marked fly before and after the removal of its fellows. The other half of the experiments was carried out in reverse, the marked fly being acclimatized and observed alone for the first half of the 40 min. period of observation. Recently emerged insects

were used in order to reduce periodicity to a minimum. In a series of fourteen experiments the mean activity of the marked fly (average 1·57 flights/min.) showed a significant increase of activity by $57 \pm 26\%$, standard error ($P < 0{\cdot}05$), in response to the presence of the other four.

In the second series of experiments, the average activity of groups of five, ten and forty flies were investigated in relation to the number in the group. Each group was acclimatized for 20 min. and then observed for a further 20 min., the average activity being compared with that of a control group of twenty treated in a precisely similar manner and run immediately beforehand. Precautions were taken to make sure that the experimental and control groups represented similar random samples of the same stock. The results are shown in Table 1. The average activity showed no significant increase with crowding between groups of five and groups of forty, activity with ten in the chamber being even slightly greater than with the larger control group.

Table 1. *Effect of crowding on activity with between 5 and 40 flies in the chamber*

No. in experimental group ...	5	10	40
No. of experiments	11	5	5
Mean activity of control group of 20 flies (flights/specimen/min.)	7·32	5·91	5·00
Difference in activity of experimental and control groups, as percentage of activity of control group (with standard error)	$+11\% \pm 12\%$	$+14\% \pm 3\%$	$+2\% \pm 3\%$

Thus, although crowding probably leads to some increase of activity at low density, at higher densities there is little or no effect. As mutual stimulation might be expected to be more effective at higher densities, this suggests that two antagonistic factors may be operating, but investigation of this matter must await further study.

These insects were very active, flights being made at the rate of about 200/min. with forty flies in the chamber, and the experiments with varied numbers of insects cover the range of total activity normally observed in the group of forty. If our original supposition is correct—that is, if mutual stimulation occurred it would result in change of activity with crowding—we may conclude that these effects are small enough not seriously to invalidate the results of experiments on the reactions of a group to sensory stimuli. The conclusions reached in this paper are subject to such mutual stimulation as does occur.

4. ADAPTATION AND THE RESPONSE TO LIGHT

Adaptation

Changes of activity resulting from changes of light are subject to adaptation. Thus the decrease of activity of a group of forty flies resulting from a decrease of light intensity (Fig. 3) was followed by a certain amount of recovery; a family of

curves showing such recovery is given in Fig. 6. This goes on for a long time, as is shown by Fig. 4. Curve *A* illustrates the activity during ½ hr. at 2600 lux followed by about 4 hr. at 5·2 lux, and finally by ½ hr. at 2600 lux again. The low level of activity reached shortly after the decrease of light showed a slow increase of about 50% in 5 hr., and when the light was returned to 2600 lux, activity became greater than at the beginning of the run. When the changes were made in the reverse order (curve *B*), activity during the long period at high light intensity showed a slow decrease.

This return of the rate of flight activity towards that existing before the change is an adaptation of population activity, and the term adaptation is used here in this sense, without prejudice as to the nature of the adaptation. Whether it arises as nervous adaptation or through some other factor not connected with the nervous system is not within the scope of this paper.

Because of these adaptive trends the change of activity following a change of stimulus must be related to a particular time interval after the change.

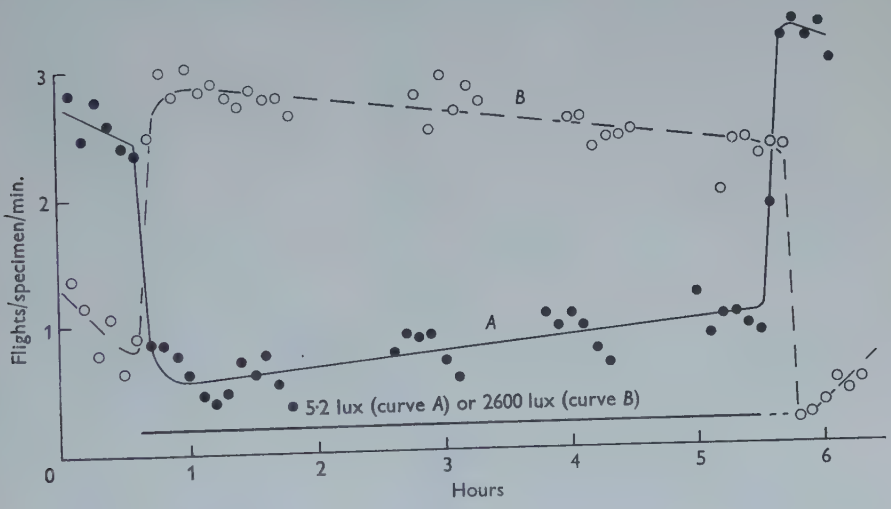

Fig. 4. Activity of a population over a period of 6½ hr. during which the light intensity was changed from 2600 to 5·2 lux and back (curve *A*) and from 5·2 to 2600 lux and back (curve *B*) showing long-term adaptive trends in each case.

The relation of activity to light intensity

Analysis of the relation between change of activity and change of light intensity in a group of insects demands that the initial state of the insects with regard to light-adaptation shall be constant. The time required for complete adaptation is so long that it is out of the question to adapt the insects fully to the initial light intensity before each change. It was found practicable to subject a group of forty flies to a convenient standard intensity of 2600 lux for ½ hr. at the beginning of each experiment and to regard them as relatively adapted to that intensity during the last 10 min. This initial period was followed by ½ hr. at an experimental light intensity,

after which the light was returned to the standard for a further ½ hr. as the beginning of the next experiment. Two such experiments are shown in Fig. 3; a number of them were commonly carried out in a day, during which the activity at the standard value would usually show a decline. This decline, an adaptation remainder so far as these short-period experiments are concerned, arises partly from the fact that the initial light intensity of 2600 lux was stronger than that maintained in the cages and in the insect chamber prior to the experiments.

To make the various experiments comparable, activity at each experimental intensity was expressed as a percentage of that during the last 10 min. of the preceding standard period. This method is not without its drawbacks, for the percentage change of activity at a given light intensity does vary with large changes in the excitability of the stock, as is shown by Fig. 4. This treatment was, however, adopted for convenience, for among the possible simple methods of comparison it gave the greatest consistency in repeated identical experiments.

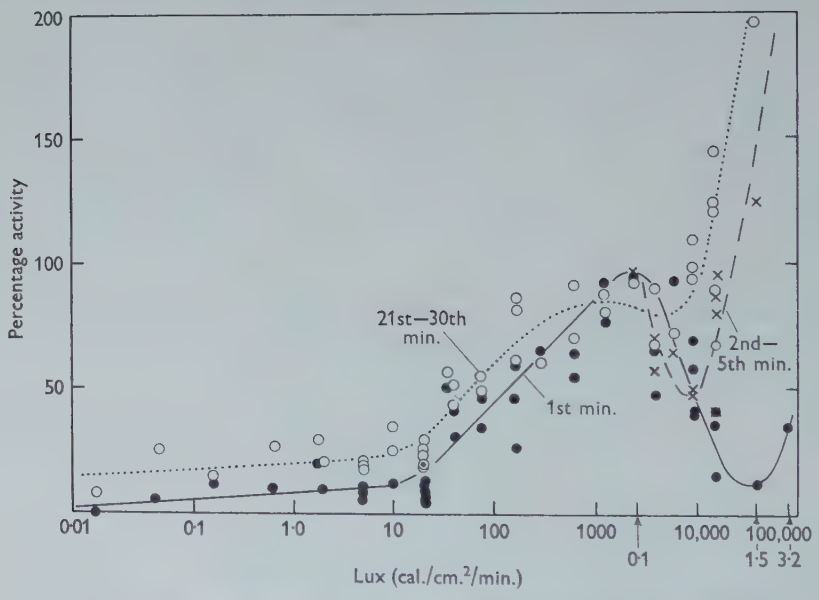

Fig. 5. Activity following a change in light intensity at various periods after the change. Activity expressed as a percentage of that prevailing during the last 10 min. of the preceding period at 2600 lux.

Curves relating the change of activity to change of light intensity are given in Fig. 5 for the periods of the first, the second to the fifth and the twenty-first to the thirtieth minutes after the change. These experiments were carried out over a range of temperatures (18·4–25·8° C.) and humidities (45–73% R.H.). Details are given in Table 2. Correlation coefficients (Fisher, 1925–50) relating the magnitude of the change of activity to these factors showed them not to be significant ($r = 0{\cdot}08$, $n = 44$, $P > 0{\cdot}05$ and $r = 0{\cdot}003$, $n = 23$, $P > 0{\cdot}05$, respectively).

Table 2. *Relation between change of activity and change of light from 2600 lux to experimental value, with temperature and humidity at which experiments were carried out*

Experimental value of light (lux)	Temperature (° C.)	Humidity (% R.H.)	Activity 21–30 min. after change (%, see text)	Experimental value of light (lux)	Temperature (° C.)	Humidity (% R.H.)	Activity 21–30 min. after change (%, see text)
	In last 10 min. of initial period at 2600 lux				In last 10 min. of initial period at 2600 lux		
0·0002	20·6	—	0·083	1·6	24·0	—	0·55
0·0008	21·6	—	0·26	3·2	19·2	68	0·61
0·0032	21·6	—	0·17	3·2	25·8	—	0·82
0·013	21·6	—	0·28	3·2	25·0	—	0·87
0·035	19·7	68	0·30	6·5	21·3	56	0·60
0·05	23·9	—	0·21	13	20·2	66	0·71
0·1	18·4	65	0·22	13	23·0	—	0·92
0·1	18·5	65	0·18	25	20·6	65	0·81
0·1	20·6	53	0·19	25	22·0	—	0·89
0·1	23·5	—	0·18	50	18·9	69	0·93
0·2	19·2	64	0·35	79	18·4	54	0·90
0·2	23·7	—	0·26	79	18·7	67	0·68
0·4	18·6	65	0·30	125	21·3	46	0·73
0·4	23·7	—	0·24	190	20·4	52	1·00
0·4	25·0	—	0·27	190	22·6	71	1·10
0·4	24·7	—	0·24	190	20·1	63	0·94
0·4	25·1	—	0·20	310	22·8	45	1·44
0·4	24·8	—	0·24	310	21·8	73	1·21
0·7	21·0	64	0·58	310	22·5	—	0·89
0·8	24·3	—	0·52	310	23·0	—	1·24
0·8	23·8	—	0·44	800	20·6	64	2·05
1·6	18·6	65	0·49	1600	21·5	61	2·92

A small amount of flight activity could be heard to occur in complete darkness, although counts could not be made. At intensities between 0·01 and 10 lux flight activity bears little relation to light. Between 10 and 2600 lux activity increases with light; the heating effects of radiation may be shown to be unimportant at this level and changes of activity are due to light alone. The extent of adaptation over this range, shown by the difference in position of the curves for the first and for the twenty-first to thirtieth minutes, is relatively small compared with the variation in activity with light intensity. Above 2600 lux, which corresponds in these experiments to a radiation intensity of 0·1 cal./cm.^{2}/min., activity shows an initial decrease followed by an increase to values greater than those to be expected from extrapolation of the part of the curve below 2600 lux. It will later be shown that these effects are due to heating.

If we regard light as one of a number of activating stimuli (Wolsky, 1933), the fact that flight activity is relatively independent of light at low intensities suggests that under these conditions other activating stimuli predominate. One of these is certainly wind, for when the insects are left in the chamber in darkness and in still air they become almost completely inactive.

5. MEASUREMENT OF ADAPTATION TO LIGHT

Principles in analysis of adaptation

It was found that while activity decreased rapidly on decreasing the light intensity, the increase of activity which followed an increase of light intensity was a much slower process.

The course of adaptation to greater and to lesser light intensities was studied in order to investigate these differences more fully. The principles employed in this analysis are as follows. The population is subjected to a given level of stimulus until the rate of activity evoked has become steady, when the population may be said to have become adapted to it. If the stimulus is then changed, the extent to which adaptation occurs to the second level can be assessed at any instant by returning the stimulus to the initial level. The curve of activity during the second period at the initial level of stimulus, when the insects are recovering from the effects of the changes, may be called a recovery curve. The difference in activity at the initial level of stimulus, as measured before and again immediately after the period at the second level, is a measure of the amount of adaptation which has taken place to the second level of stimulus. The time course of adaptation may be plotted by carrying out a series of experiments with different values for either the magnitude or duration of the second level of stimulus. Duration has been varied in these experiments.

Adaptation to high light intensity

To analyse the course of adaptation to high light intensity (Fig. 6) the insects were adapted first of all to a low intensity (20 lux) until the activity became relatively constant. They were then subjected to varying periods at a higher intensity (2600 lux), after which the low intensity was applied once more. The second period of low intensity is a test stimulus and the family of recovery curves of activity during this period show the following features: a short period of high light intensity results in an initial depression, a rebound, small in both magnitude and duration, during this second period of low intensity, while a longer period of high intensity results in a greater and a longer depression. The activity at any particular moment during this test period differs from that at the end of the initial period at low intensity by an amount which is a measure of the adaptation to the higher light intensity at that moment. This adaptation to the experimental value is maximal at the first instant of the test period, after which there is re-adaptation back to the initial level. The initial values of a family of these recovery curves form an adaptation curve (Fig. 6, broken line), relating the extent of adaptation to the duration of the adapting stimulus. This adaptation curve is somewhat diagrammatic, as it is compounded from experiments carried out on different occasions with stock not always of identical age and sensitivity. Nevertheless, it can be seen that the most rapid part of adaptation to high light intensity is complete in 20 min. or $\frac{1}{2}$ hr.

Adaptation to low light intensity

Adaptation to low light intensity was investigated by using the same intensities in the reverse order. The effect of periods of low light of varying duration was studied by observing the activity during the following periods of high light. This family of curves (Fig. 7) is not a mirror image of the family of curves in Fig. 6; there is no obvious rebound. Indeed, the two sets appear at first sight to be remarkably

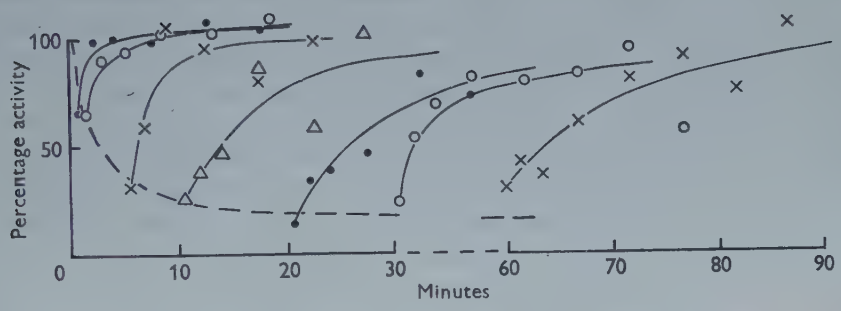

Fig. 6. Adaptation to high light intensity (i.e. family of recovery curves at 20 lux after varied periods at 2600 lux during which the activity was much greater). The broken line, passing through the initial point of each curve, shows the time course of adaptation to 2600 lux. Activity expressed as a percentage of that prevailing during the last 10 min. of the initial period at 20 lux, time as minutes elapsed since the end of this initial period.

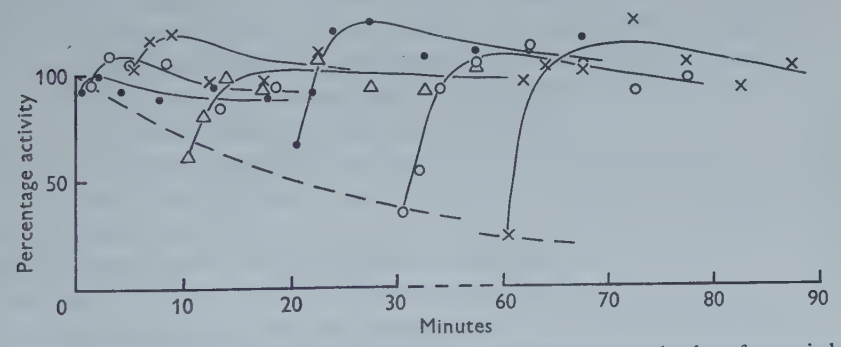

Fig. 7. Adaptation to low light intensity (i.e. family of recovery curves at 2600 lux after varied periods at 20 lux during which the activity was much less). Activity expressed as a percentage of that prevailing during the last 10 min. of the initial period at 2600 lux, time as minutes elapsed since the end of this initial period.

similar. After a period at low light intensity the activity of the insects in the first few minutes shows only a slow rise to a level a little higher than that prevailing initially.

This persistence of a state of low activity is not the result of complete lack of activity or akinesis in a certain proportion of the members, for they are all more torpid. It was necessary to ascertain whether this state of low activity might be due entirely to change in light intensity, or whether heat might be an important additional factor. Heating causes an initial depression of activity, and although but

little heating is involved at 2600 lux it seemed desirable to investigate the matter. Experiments on the increase of activity with increase of light from 10 lux to values between 40 and 6400 lux all showed the same slow increase of activity with increase of light, however, and as heating effects were very small indeed at these lower light intensities, we may conclude that the state of persistent low activity arises entirely from changes of light intensity.

Peaks of activity can be traced in the recovery curves at times varying from 3 to 15 min. after the resumption of high light intensity. After this peak in each recovery curve, the trend becomes the mirror image of that of the recovery curves following a period of high light intensity. The initial parts of the recovery curves show that during the preceding low level of stimulation, when activity has been low but rising, the state of persistent low activity had been setting in progressively.

Although the persistence of the state of activity existing before the change is most noticeable in the recovery from a low light intensity, there is also a short lag following the reverse change. Activity commonly declines over a period of from 10 sec. to $2\frac{1}{2}$ min. when light intensity is reduced.

6. THE EFFECT OF STRONG RADIATION

The nature and cause of the reactions to strong radiation

When the light intensity was changed from the standard 2600 to 4100 lux, and above (Fig. 8), there was an initial drop in flight activity for 1–4 min. followed by an increase, usually to a level above that expected from extrapolation of the curve relating activity to light at lower intensities (Fig. 5). There are always, at any one time, a number of insects remaining still or carrying out grooming movements, and during the initial fall in activity this proportion increases so that for some few seconds almost the whole population exhibits these phases of activity. The fact that inhibition of activity is followed by a rise to a final level of activity well above that prevailing before the increase of light shows that at least two reactions must be concerned. We may call the first an inhibition, beginning immediately after the change, and the second an activation, setting in a little later. These reactions may be seen from Fig. 5 to become apparent first above 2600 lux, although the variability of activity in each individual run precludes their detection in any single experiment below about 4100 lux. The minimum intensity of 2600 lux, equivalent to 0·1 cal./cm.^{2}/min., was sufficient to heat the insects by about 1° C., which suggests that the inhibition and activation might well result from the rise in temperature.

If, however, the reactions were due to the perception of illumination the threshold would probably be decreased by dark-adaptation. Thirty-eight experiments had been concerned with the effects of an increase of light to various intensities up to 2600 lux with insects dark-adapted to varying degrees. The increase of activity with increase of light was always delayed by the persistence of a state of low activity, but an actual reduction of activity during the first minute or two only occurred when the intensity was raised to 4100 lux or above, whatever the initial

value had been. This strongly suggests that the effects are not due to light intensity perceived as illumination.

Experiments were therefore carried out to separate the effects of light and heat. Precautions were necessary, for the inhibition is very susceptible to adaptation. In young insects some nine runs on various occasions have shown that the identical reactions can be evoked time and time again, but in older stock (Fig. 8) the general level of activity declines very rapidly and three or four 10 min. periods at a higher radiation strength results in the stock no longer showing inhibition, although activation may still occur. The transitory inhibition may also be lost if the insects have become adapted to heat by crawling close to an exposed electric light bulb in a breeding cage during cold weather. Accordingly, each experiment took the form of a particular experimental change, followed shortly afterwards by the change from 2600 to 16,000 lux as a control, to make sure that the stock was still capable of exhibiting both reactions. Excessive heating effects were avoided.

In one series of experiments strong light intensities were applied while the heating effect was increased by removing the water-bath from beneath the bulb at intervals. When carried out at 6400 lux, inhibition and activation occurred. The average activity change of three experiments carried out in the course of one run is shown in Fig. 9. Two further runs of three successive experiments each gave very similar results. Removal of the water-bath at this intensity caused an increase of the heating effect from 0·25 to 0·47 cal./cm.^{2}/min. for an increase of light of about 15%. As this change in light intensity has in itself a negligible influence in the absence of substantial changes in heat, the effect must be due to heat alone.

To confirm this, a range of intensities of infra-red radiation from an electric radiator fitted with screens was allowed to fall on the chamber at intervals, light intensity being kept at 2600 lux throughout. Three runs comprising ten experiments with infra-red ranging between 0·25 and 0·5 cal./cm.^{2}/min. produced transitory inhibition, sometimes followed by activation depending upon the stock used. The effect of the addition of 0·5 cal./cm.^{2}/min. of infra-red compared with that of an increase of illumination to 16,000 lux (equivalent to 0·63 cal./cm.^{2}/min.) is shown in Fig. 10. As the illumination provided by the radiator represented only a negligible fraction of that provided at the same time by the bulb behind the water-bath, we may conclude that the inhibition must again have been due solely to the heating effect of the radiation.

The reaction to various ways of heating

The heating effect of the radiation in these experiments may operate in various ways. The celluloid surfaces of the insect chamber become heated by absorption of radiation of longer wave-lengths and will warm particularly the tarsi and legs; the warmed celluloid surfaces raise the temperature of the layers of air immediately adjacent to them, which will warm each insect as a whole; and direct absorption of radiation by the insects themselves will cause an increase of temperature in the main body of the insects more than in the appendages. The insects may or may not react to radiation heating in the same way as to an increase of air temperature. As

the organs for the perception of heat may well be localized, it is of interest to know whether there is any difference in the response according to the way in which the heating is applied.

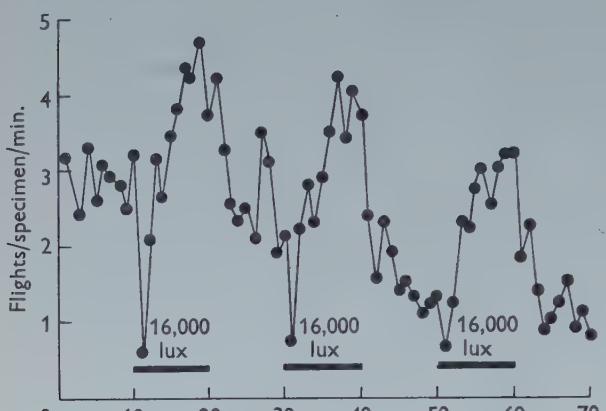

Fig. 8

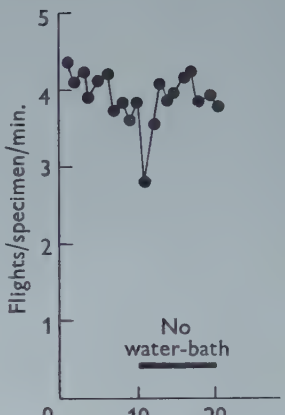

Fig. 9

Fig. 8. Effect of the change from 2600 to 16,000 lux, showing inhibition and activation, and the decline in response with repeated stimuli.

Fig. 9. Effect of removing water-bath from beneath bulb, at illumination strength of 6400 lux. Average of three experiments.

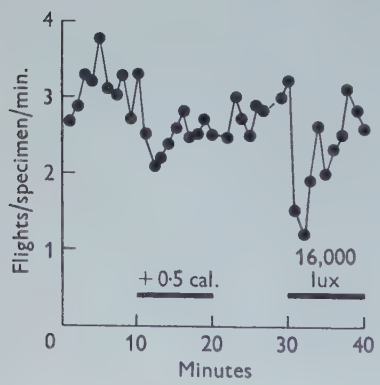

Fig. 10

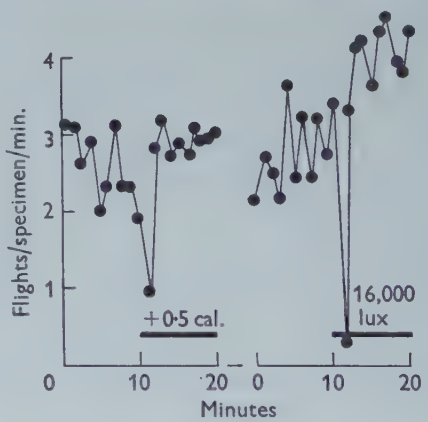

Fig. 11

Fig. 10. Effect of addition of 0·5 cal./cm.²/min. of infra-red radiation from electric radiator to a light intensity of 2600 lux compared with effects of change from 2600 to 16,000 lux, in the usual celluloid chamber.

Fig. 11. Effect of addition of 0·5 cal./cm.²/min. of infra-red radiation to light intensity of 2600 lux, compared with the effect of change from 2600 to 16,000 lux in chamber of nylon mesh.

Measurements of temperature profiles in the insect chamber showed the control change from 2600 to 16,000 lux to be associated with a maximum increase of temperature of 1·75, 1·6 and 0·6° C. close against the surface of the chamber and at distances of 2 and 5 mm., respectively. These distances correspond roughly to the

heights of the upper and lower surfaces of a fly. The temperature excess attained by the thorax of the insect by virtue of its own absorptivity lay between 3 and 7° C. (Digby, 1955).

The effect of a heated surface was eliminated by using a new insect chamber, similar in size but with the celluloid replaced by a fine nylon mesh, transmitting all but about 7% of the light and heat. When fitted in place of the celluloid chamber with the shutters adjusted to obtain a slight positive air pressure, most of the air passed straight through the chamber from end to end, while a part escaped through the meshes, causing the heating of the surface to be quite negligible. In this chamber inhibition and activation occurred as before when a range of values of infra-red radiation was applied at a constant light intensity of 2600 lux (Table 3). Fig. 11 shows the effect of the addition of 0·5 cal./cm.^{2}/min. of infra-red. The effects of radiant heat can therefore be produced when the insects are on an unheated surface.

Heating of the surface itself was investigated with a third type of insect chamber, again similar but with the upper and lower surfaces and the back formed by electrical resistance mats heavily insulated by shellac and with the ends and near side made of nylon mesh. The right-hand end was completely blocked, the air flowing into the left-hand end at 0·5 m./sec. and passing out through the spaces between the wires and through the nylon mesh in front, allowing the surfaces to be heated by an electric current with relatively slight heating of the air inside the chamber. Radiative heating was slight. Again, an appropriate amount of heating gave results very similar to those obtained in the control celluloid chamber (Table 3). Effects similar to those obtained with the controls in the celluloid chamber with a maximum surface temperature increase of 1·75° C. were, however, only produced in the resistance mat chamber with a surface temperature increase of 12° C. Measurement of the temperature profile in this latter case showed air temperatures at 2 and 5 mm. above the surface to have increased by 3·0 and 6·5° C., respectively, which is comparable to the temperature excess developed by the insects in the control chamber. This suggests that perception of heat may depend on temperature changes in the main body of the insect rather than in the legs or tarsi.

The effects of air temperature were studied by fitting a heating element across the wind channel, sufficiently far from the insect chamber for the amount of heat received by radiation to be very slight. The effect of an increase of air temperature is to warm the main body of the insect and the appendages by a similar amount. A fine thermocouple showed that the temperatures across the channel were relatively uniform and reached to within 1·0° C. of equilibrium in less than a minute. Experiments were carried out at 2600 lux, flies being adapted to this light intensity and to a temperature of 20° C. for $\frac{1}{2}$ hr. or more before each experiment. Heating of the air to the appropriate amount caused inhibition and activation in a way similar to that in the control celluloid chamber (Fig. 12, Table 3). This occurred with an increase of air temperature of 5–10° C., of the same order as the temperature excess developed by insects at 16,000 lux in the controls. This suggests that these reactions are due primarily to an increase of temperature of the insects, rather than to the perception of radiant heat as such.

Table 3. *Effect on activity of heating the population in various ways*

Method of heating	Amount of heating	Total no. of experiments	No. showing no effects	No. showing inhibition followed by activation not exceeding initial level	No. showing inhibition followed by activation to above initial level
Nylon mesh chamber (cool surface). Increase of infra-red radiation by	0·3 cal./cm.²/min.	9	9	—	—
	0·5 cal./cm.²/min.	12	7	5	—
	1·0 cal./cm.²/min.	3	—	—	3
Resistance mat chamber (warm surface). Increase of surface temperature by	2° C.	3	3	—	—
	Up to 12° C.	6	1	5	—
	Above 12° C.	6	—	—	6
Celluloid chamber. Increase of air temperature by	5° C.	6	1	5	—
	10° C.	6	1	6	—
	15° C.	4	—	—	4

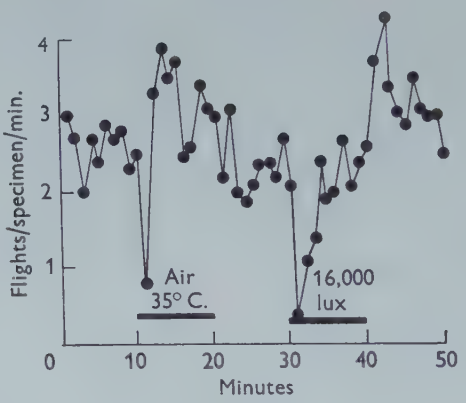

Fig. 12. Effect of an increase of air temperature from 20 to 35° C. and of an increase of light intensity from 2600 to 16,000 lux, in a population of young insects.

7. DISCUSSION

The study of adaptation has been very largely neglected in the field of the relations between insect activity and environmental factors. Adaptation is, however, commonly observed. Thus the curves of activity in alternating light and darkness given by Bentley, Gunn & Ewer (1941) for the beetle *Ptinus tectus* show adaptive trends similar to those demonstrated for *Calliphora*, but with a much longer time scale. In their experiments activity would appear to take an hour or two to reach the extreme value following each change, and the adaptive trend which follows is clearly only partly complete after a further 10 hr. These trends perhaps correspond to the long-term changes illustrated in Fig. 4 of this paper. Broadbent (1949), working with the aphids *Myzus persicae* and *Brevicoryne brassicae*, found that after turning on the light, following a period of darkness during which the insects had become quiescent, activity increased slowly. Activity at a standard low light intensity was low when the light had been reduced from a higher value, but was high, and was

followed by a decrease, when light had been increased from a lower value. These results suggest similarity of behaviour with *Calliphora*. Adaptation also occurs in the relation of activity to temperature, quite apart from the transitory inhibition caused by rapid heating as described in this paper. The differences between the curves of activity of *Lucilia* (Nicholson, 1934) and *Ptinus* (Gunn & Hopf, 1942) at constant and at rising or falling temperatures are a result of adaptive trends, for in this type of experiment the two groups differ in that they have been adapted either to the temperature of observation, or to lower or higher temperatures, respectively. The faster the temperature is raised or lowered, the greater is the difference between that to which the insect is adapted and that to which it is exposed. Such procedure, however, gives very little information on the rate of adaptation to the change of stimulus.

Such adaptive trends are of significance in the ecology of an insect, in that the influence of a particular environmental factor on activity is incompletely defined unless account is taken of the rate of change of the factor as well as its absolute value.

It was shown that a state of low activity induced by a low light intensity tended to persist into a subsequent period of higher light intensity. This persistent state of low activity would appear to have a resemblance to reflex immobilization or akinesis. Reflex immobilization is brought about by powerful inhibitory stimuli, but the effects described here are induced by reduction in the activating stimulus of light intensity. As, however, it is reasonable to suppose that in each individual *Calliphora* a certain background of nervous inhibition is always present, partly at least provided by the stimulus of contact between the legs and the substratum (Fraenkel, 1932; Hollick, 1940), reduction of the activating stimulus of light will, in each insect, alter the balance between activating and inhibiting stimuli in favour of the latter, so that inhibiting stimuli are relatively more powerful at lower light intensities. The state of persistent low activity in *Calliphora* would, therefore, be essentially similar in origin to reflex immobilization, from which it may be considered to differ only in degree.

It is to be stressed that adaptation in this paper refers to adaptation of population activity, and not to that of the sensory end-organs responsible for receiving the operative stimulus.

The transitory inhibition caused by the sharp increase of heat of the appropriate amount was not encountered by Nicholson (1934) or by Gunn & Hopf (1942) in their experiments with blowflies and with *Ptinus*; they would hardly be expected as the rates of temperature increase employed ranged between 3 and 14° C. per hour. The reactions appear in *Calliphora* at rates of 5° C. per minute and upwards. Such sharp temperature changes will often be encountered in the field, and this transitory inhibition produced by heating may well be one factor concerned in the way in which *Calliphora* and other insects bask in the sunshine. Other factors are doubtless also involved, for instance a decrease of activity is caused by increase of temperature above an optimum. Basking involves both orientation and reduction of activity. The former has received considerable attention; thus the orientation assumed by the locust is determined by the internal temperature (Volkonsky, 1939) and it is

adjusted as a result of responses from organs receptive to radiant heat (Slifer, 1951). The transitory reduction in activity in *Calliphora* is not obviously associated with orientation and is evoked by an increase of air temperature as well as by radiant heat, the radiation intensity required in the latter case being such as to suggest that the gross change of temperature of the insect is the causative factor.

8. SUMMARY

1. The spontaneous flight activity of a population of the blowfly *Calliphora erythrocephala* has been studied in relation to light and radiant heat in a small wind tunnel.

2. Flight activity occurs at a low level in the dark under these conditions, and is practically independent of light up to 10 lux. Activity increases sharply with light between 10 and 2600 lux, before heating effects become important.

3. Population activity shows adaptation to light intensity, and a method of analysis is described. The rate of increase of activity following an increase of light is much slower than is the reverse change; the state of low activity at low light intensities tends to persist. Complete adaptation would take at least a number of hours.

4. Heating, which accompanies strong light intensities, causes initially a momentary decrease in activity, usually followed by an increase. Similar reactions occur when the heat is applied in several different ways.

I am indebted to Professor Sir Alister C. Hardy, F.R.S., for encouragement and facilities for this work which was begun at the Department of Zoology and Comparative Anatomy, Oxford, supported by a grant from the Agricultural Research Council. My thanks are also due to Mr F. R. Petherbridge, Mr J. H. Stapley and Mr D. W. Wright at the School of Agriculture, Cambridge, where some initial experiments had been made, and to Dr A. G. Hamilton at St Thomas's Hospital Medical School where the work was continued. I am particularly grateful to my wife for her help at many stages.

REFERENCES

ATKINS, W. R. G. & POOLE, H. H. (1936). Photoelectric measurements of the Luminous Efficiency of Daylight. *Proc. Roy. Soc.* B, **121**, 1–17.

BENTLEY, E. W., GUNN, D. L. & EWER, D. W. (1941). The biology and behaviour of *Ptinus tectus* Boie. (Coleoptera, Ptinidae), a pest of stored products. I. The daily rhythm of locomotory activity, especially in relation to light and temperature. *J. Exp. Biol.* **18**, 182–95.

BLACKWELL, M. J. (1954). Five years continuous recording of total and diffuse solar radiation at Kew Observatory. *Met. Res. Publ.* no. 895. London: Air Ministry.

BROADBENT, L. (1949). Factors affecting the activity of alatae of the aphids *Myzus persicae* (Sulzer) and *Brevicoryne brassicae* (L.). *Ann. Appl. Biol.* **36**, 40–62.

DAVIES, W. M. (1935). Studies on aphids infesting the potato crop. III. Effect of variation in relative humidity on the flight of *Myzus persicae* Sulz. *Ann. Appl. Biol.* **22**, 106–15.

DAVIES, W. M. (1936). Studies on the aphides infesting the potato crop. V. Laboratory experiments on the effect of wind velocity on the flight of *Myzus persicae* Sulz. *Ann. Appl. Biol.* **23**, 401–8.

DIGBY, P. S. B. (1955). Factors affecting the temperature excess of insects in sunshine. *J. Exp. Biol.* **32**, 279–98.

FISHER, R. A. (1925–50). *Statistical Methods for Research Workers.* Edinburgh: Oliver and Boyd.

FRAENKEL, G. S. (1932). Untersuchungen über die Koordination von Reflexen und automatisch-nervosen Rythmen bei Insekten. I. Die Flugreflexe der Insekten und ihre Koordination. *Z. vergl. Physiol.* **16**, 371–93.

FRAENKEL, G. S. & GUNN, D. L. (1940). *The Orientation of Animals.* Oxford: Clarendon Press.

FREEMAN, J. A. (1945). Studies in the distribution of insects by aerial currents. *J. Anim. Ecol.* **14**, 128–54.

GISLÉN, T. (1948). Aerial plankton and its conditions of life. *Biol. Rev.* **23**, 109–26.

GUNN, D. L. & HOPF, H. S. (1942). The biology and behaviour of *Ptinus tectus* Boie. (Coleoptera, Ptinidae), a pest of stored products. II. The amount of locomotory activity in relation to experimental and to previous temperatures. *J. Exp. Biol.* **18**, 278–89.

HARDY, A. C. & MILNE, P. S. (1937). Insect drifts over the North Sea. *Nature, Lond.*, **139**, 510.

HARDY, A. C. & MILNE, P. S. (1938). Studies in the distribution of insects by aerial currents. *J. Anim. Ecol.* **7**, 199–229.

HOLLICK, F. S. J. (1940). The flight of the Dipterous fly *Muscina stabulans* Fallen. *Phil. Trans.* B, **230**, 357–90.

JOHNSON, C. G. (1954). Aphid migration in relation to weather. *Biol. Rev.* **29**, 87–118.

NICHOLSON, A. J. (1934). The influence of temperature on the activity of sheep blowflies. *Bull. Ent. Res.* **25**, 85–99.

RAINEY, R. C. & WALOFF, Z. (1948). Desert locust migrations and synoptic meteorology in the Gulf of Aden area. *J. Anim. Ecol.* **17**, 101–12.

SLIFER, E. H. (1951). Some unusual structures in *Locusta migratoria migratoriodes* and their probable function as thermoreceptors. *Proc. Roy. Soc.* B, **138**, 414–37.

VOLKONSKY, M. (1939). Sur la photo-akinèse des acridiens. *Arch. Inst. Pasteur Algér.* **17**, 194–220.

WALOFF, Z. (1946). A long range migration of the desert locust from southern Morocco to Portugal, with an analysis of concurrent weather conditions. *Proc. R. Ent. Soc. Lond.* A, **21**, 81–4.

WOLSKY, A. (1933). Stimulationsorgane. *Biol. Rev.* **8**, 370–417.

EFFECT OF THE BLACK SNAKE TOXIN ON THE GASTROCNEMIUS-SCIATIC PREPARATION

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When the toxin of the black snake (*Walterinnesia aegyptea*) was injected subcutaneously into albino rats, one of the common symptoms which appeared was the paralysis which takes place in the limb near the site of injection and extends to other limbs just before death.

Such observations led us to examine the changes produced in a stimulated gastrocnemius-sciatic preparation of *Bufo* to show the effect of the toxin and the site of its action.

No work had been done on the action of the black snake toxin on the nerve-muscle preparation. Yet much work has been published about such data for other poisonous snakes.

Lauder, Brunton & Fayrer (1874) were the first to demonstrate a curare-like action of cobra venom in dogs.

Epstein (1930) observed complete loss of excitability of voluntary muscles brought about by cape cobra venom and Kellaway & Holden (1932) demonstrated the direct action of venom on muscles.

Sarker (1951) has reported the action of Indian cobra venom both on the muscle and neuromuscular junction, the paralysis of the latter setting in much earlier and with smaller concentrations of the venom.

The aim of this work was to study the following points:

(1) The effect of the toxin of the black snake on the contraction height of gastrocnemius muscle when stimulated directly and/or indirectly.

(2) The site of action of the toxin.

(3) The mechanism of action of the toxin.

METHODS

The toxin was prepared according to methods previously described (Mohamed & Zaki, 1956).

The gastrocnemius-sciatic preparation of the toad *Bufo* was employed.

A muscle trough was divided into two chambers by interposing a paraffin wall across the trough having a small groove for the transit of the nerve across the wall. The trough was so constructed that the bathing solution could be changed readily. Special care was necessary to avoid even small injuries to muscle fibres during dissection. Frog Ringer's fluid was used. The solution was freshly prepared for each day's experiment from stock solutions. Curare was used throughout in

concentration of 1×10^{-4}. With this concentration complete neuromuscular block was obtained and so direct stimulation could be applied to a curarized muscle.

The sciatic nerve was stimulated through submersible electrodes by supramaximal rectangular pulses of 300 μ sec. duration delivered at a rate of six per minute from an electronic square wave stimulator. The frequency was such as to keep the muscle contracting with no fatigue and so a constant contraction height was obtained. The duration used was the shortest effective duration so as to avoid repetitive excitation in nerve and summation in muscle.

A spring-loaded lever was used to record the contraction on a slowly revolving drum. The preparation was always stimulated for $1\frac{1}{4}$–$1\frac{1}{2}$ hr. before starting an experiment to allow stretching of the muscle to become complete, and for the preparation to settle down to a steady level of contraction.

In control experiments the gastrocnemius continued to contract for at least 3–4 hr. without appreciable decline.

RESULTS

A. *Site of action of the toxin*

(1) *Effect of toxin on the nerve*

The nerve of the gastrocnemius-sciatic preparation was immersed in toxin solution and the muscle in Ringer's solution. The soaking of the nerve in 1/5000 toxin produced no change in the contraction of muscle over the next 200 min.

This shows that the toxin has no effect on the excitability of the nerve.

(2) *Effect of toxin on the muscle*

The gastrocnemius muscle was immersed in the toxin and the nerve in Ringer's solution.

Using three different concentrations of toxin 1/5000, 1/10,000 and 1/20,000 the time required to cause paralysis of the muscle by the solutions, as determined by the response both to direct and indirect stimulation was noted.

With the lower concentrations 1/10,000, 1/20,000 of toxin the latency was increased in duration and the rate of paralysis was slowed. With the very low concentration of 1/10,000 no change was detected in the preparation run for about 2 hr.

Results of the effects of three concentrations are shown in Table 1.

It will be noticed from the table that at the time when the stimulation of the nerve fibres fails to evoke any contraction the muscle still shows some contraction on direct stimulation. This means that although the toxin has slight depressant action on the contractility of the muscle, yet its main action is not direct on the muscle but is on the neuromuscular junction.

(3) *Post-tetanic potentiation*

Brown & v. Euler (1938) showed that in normal and partially curarized muscle a short tetanus of the motor nerve increased the tension developed in the succeeding single twitches.

Table 1. *Action of the black snake toxin on the toad gastrocnemius-sciatic preparation*

(The toxin is in the muscle chamber.)

Exp. no.	Concentration of toxin	Time required for no contraction of muscle when stimulated (min.)	
		Indirectly	Directly
1	1/5,000	55	195
2		65	180
3		65	185
1	1/10,000	105	250
2		90	280
3		95	245
1	1/20,000	210	390
2		195	370
3		180	330

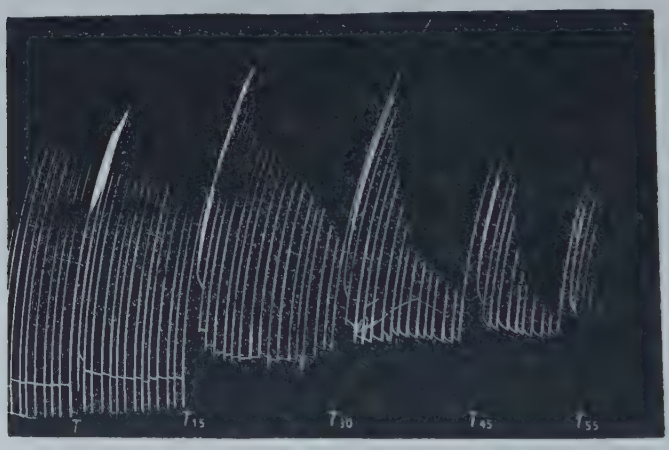

Fig. 1. Post-tetanic potentiation in gastrocnemius-sciatic preparation. Indirect stimulation six per min. At points T, T_{15}, T_{30}, T_{45} and T_{55} the rate of stimulation was increased to 50/cyc./sec. for 10 sec.

After the addition of toxin to the muscle bath (1/5000 concn.), the post-tetanic potentiation was tested at different intervals during the progress of paralysis (Fig. 1). At (T), before the addition of toxin, the post-tetanic potentiation was in the ratio 5:4. At (T_{15}), 15 min. after addition of the toxin, the post-tetanic potentiation was in the ratio of 3:2. At (T_{30}), 30 min. after the addition of toxin, the potentiation was in the ratio 4:1. At (T_{55}), 55 min. after the toxin, the potentiation was in the ratio 9:1.

The degree of potentiation increases as the paralysis progresses.

As Brown and v. Euler (1938) showed that the post-tetanic potentiation was essentially a muscular phenomenon and was present on direct stimulation of the

fully curarized or denervated muscle, it is not surprising that it should persist in the case of poisoning by black snake toxin, and this fact offers further evidence of the lack of strong direct action on the muscle, and that the main effect of the toxin is on the neuromuscular junction.

B. *Mechanism of the neuromuscular block produced by black snake toxin*

The neuromuscular block may be either a result of

(*a*) reduction in the sensitivity of the end-plate to the depolarizing action of acetylcholine, i.e. curare-like action; or

(*b*) change in the amount of the acetylcholine liberated at the nerve ending.

These possibilities were examined by means of the following tests:

(1) Genesis of tetanus.
(2) Effect of potassium chloride.
(3) Effect of prostigmine.
(4) Effect of acetylcholine.

As 1/50,000 concentration of toxin can be relied upon to produce paralysis regularly in a convenient time, this dose has been used for all experiments.

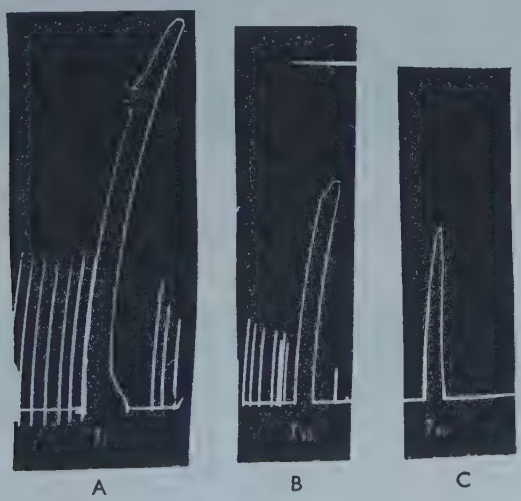

Fig. 2. Gastrocnemius-sciatic preparation. Indirect stimulation at 50 cyc./sec. for 30 sec. at T_{15}, T_{30} and T_{45}.

METHODS AND RESULTS

(1) *Genesis of tetanus*

When the sciatic nerve is stimulated at 50 cyc./sec. there is a rapid build-up of muscle tension in the first 5–10 sec. and the tetanus is then well maintained.

After partial paralysis with the toxin the response to tetanus remains perfectly normal in shape, but with declining amplitude as long as poisoning is prolonged. Figs. 2A–C show 30 sec. tetanus at different stages of poisoning. In a typical

experiment with a muscle in which curare had reduced the sciatic tension by 40%, stimulation of the sciatic nerve at 50 cyc./sec. for 30 sec. resulted in an initial strong contraction followed by a rapid fall in the tension. Thus no tetanus was produced.

(2) *Effect of potassium chloride*

It is a well-known fact that KCl has an anticurare action (Wilson & Wright, 1936). When added to the muscle-bath while the muscle is partially curarized it causes immediate recovery. The action is a maintained one. In the present experiments KCl added to the bath in concentrations of 2–15 mg./ml. produced in normal and poisoned preparations very rapid depression. This shows that while KCl improves the condition of a muscle acted upon by curare, yet it depresses the condition of a muscle acted upon by the black snake toxin.

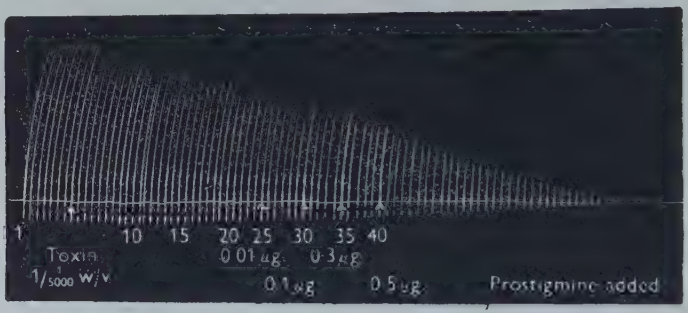

Fig. 3. Lack of beneficial effect of prostigmine upon the paralysis caused by black snake toxin. Prostigmine 0·01 μg./ml. added 25 min. after toxin. 0·1 μg./ml. added 30 min. after toxin. 0·3 μg./ml. added 35 min. after toxin. 0·5 μg./ml. added 40 min. after toxin.

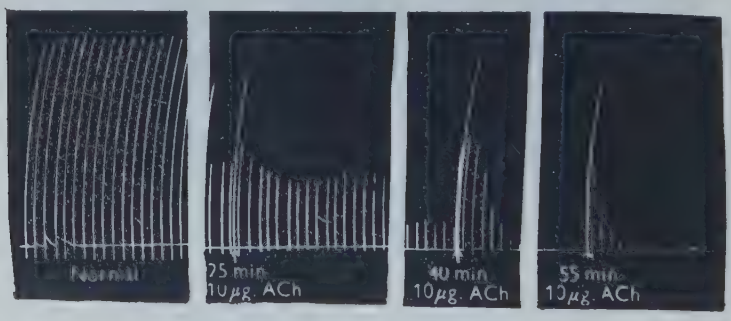

Fig. 4. Effect of acetylcholine 10 μg./ml. added to muscle chamber 25, 40 and 55 min. after addition of 1:5000 toxin solution.

(3) *Effect of prostigmine*

The blocking action of curare at the end-plate region can be overcome by administering the anticholinesterase prostigmine. If prostigmine is added to a curarized muscle after the contraction height has diminished, gradual recovery

takes place and the normal contraction height is retained. In a typical experiment (Fig. 3), after 25 min. paralysis, the effect of successive doses of 0·01, 0·1 and 0·3 mg. prostigmine is shown. The low dosage produced no effect and the contraction height remained constant in the following twitches up to 5 min. Larger amounts of prostigmine did not hinder the progress of the paralysis and there was gradual fall in the contraction height.

(4) *Effect of acetylcholine*

The neuromuscular block produced by curare results from its action on the motor end-plates which become insensitive to the acetylcholine released from the motor nerve endings (Dale, Feldberg & Vogt, 1936). During the neuromuscular block caused by black snake toxin, however, the motor end-plates remain sensitive to acetylcholine (10 μg.) as shown by the Fig. 4.

DISCUSSION

Since the gastrocnemius muscle responds when stimulated through the nerve when this is treated with the black snake toxin and the contractility remains nearly the same, it may be concluded that the toxin has no paralysing action on the nerve. When the solutions are interchanged, i.e. when the muscle is dipped into the toxin, it gradually goes into paralysis. The marked difference in time required for no contraction with direct stimulation as compared with stimulation through the nerve suggests that the black snake toxin acts mainly on the neuromuscular junction. That the muscle still shows the phenomenon of post-tetanic potentiation after being immersed in the toxin is further evidence for the neuromuscular effect of the toxin (Brown & v. Euler, 1938).

The mechanism by which the toxin produces block is entirely different from the mechanism by which curare does so. The normal type of tetanic response and the lack of response to anti-curare drugs suggest that the preparation partially poisoned with toxin contains some motor units which are susceptible to stimulation.

The end-plate is normally sensitive to acetylcholine, but the output of acetylcholine from the nerve endings is greatly diminished due to the presence of the toxin. It may be that the power to synthesize acetylcholine by the nerve endings would gradually decline, until the amount of acetylcholine fell below the threshold needed to activate the end-plate. A diminished acetylcholine output due to increased cholinesterase activity, and hence greater breakdown can be excluded, since prostigmine, a cholinesterase inhibitor, does not improve the condition of the muscle.

The release of acetylcholine may be decreased perhaps by a permeability change in the nerve endings. This is now under examination in our laboratory by a wide study of the effect of increase and decrease of the different cations in Ringer's solution. It may be that the toxin hinders the entry of Na ions into the nerve terminal and so prevents the equivalent efflux of acetylcholine ions from the interior of the nerve endings (Fatt & Katz, 1952).

SUMMARY

1. Black snake toxin produces irreversible paralysis of the isolated toad gastrocnemius-sciatic preparation.

2. The rate of paralysis is slowed by lowering the concentration of toxin.

3. The paralysis produced by the black snake toxin is due to neuromuscular block. Conduction in the nerve is unaffected and the muscle is only slightly affected.

4. The neuromuscular block produced by the toxin differs from that of curare in that tetanus is well maintained and that paralysis is unaffected by prostigmine or potassium.

5. The neuromuscular block produced by the toxin differs from that of curare in that the motor end-plates remain sensitive to acetylcholine.

REFERENCES

Brown, G. L. & v. Euler, U. S. (1938). Post-tetanic potentiation in normal and partially curarized muscle. *J. Physiol.* **93**, 39–45.

Dale, H. H., Feldberg, W. & Vogt, N. (1936). Effect of curare on neuromuscular transmission. *J. Physiol.* **86**, 353–59.

Epstein, D. (1930). Effect of cobra venom on the excitability of skeletal muscles. *Quart. J. Exp. Physiol.* **20**, 7–11.

Fatt, P. & Katz, B. (1952). The effect of sodium ions on neuromuscular transmission. *J. Physiol.* **117**, 203–11.

Kellaway, C. H. & Holden, H. F. (1932). Mechanism of action of cobra venom on skeletal muscles. *Aust. J. Exp. Biol. Med. Sci.* **10**, 167–72.

Lauder, T. L., Brunton, W. & Fayrer, J. (1874). The effects of cobra venom on the muscles of the dog. *Proc. Roy. Soc.* **22**, 68–71.

Mohamed, A. H. & Zaki, O. (1956). Biochemical and physiological studies of the purified toxin of *Walterinnesia aegyptea*, 'The Egyptian black snake'. *J. Exp. Biol.* **33**, 502–7.

Sarker, N. K. (1951). Action mechanism of cobra venom, cardiotoxin and allied substances on muscle contraction. *Proc. Soc. Exp. Biol. Med., N.Y.*, **78**, 469–71.

Wilson, A. T. & Wright, S. (1936). Anticurare action of potassium chloride. *Quart. J. Exp. Physiol.* **26**, 127–32.

THE EFFICIENCY OF ADAPTIVE STRUCTURES IN THE NYMPH OF *RHITHROGENA SEMICOLORATA* (CURTIS) (EPHEMEROPTERA)

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INTRODUCTION

Rhithrogena semicolorata (Curtis) (Fig. 3) has long been known to inhabit fast-flowing streams, but there have been hardly any critical observations on its clinging properties in torrents. It is remarkable in that all of its seven pairs of gill lamellae overlap one another and form what has been regarded as a sucker (Percival & Whitehead, 1929) or an adhesive organ functioning on the principle of a 'Venturi' tube (Hora, 1930). If the gills act as a sucker they could only be effective on a fairly smooth surface. It thus seemed reasonable to expect that the animal would tend to have an optimal substrate on which it would obtain the best hold using both sucker and claws. It was in order to find this postulated optimal surface and to attempt to assess the true value of the gills as a structural adaptation to currents that the experiments described below were carried out. In order to obtain a comparison with a mayfly nymph of similar structure and same habitat, yet not possessing overlapping sucker-like gills, *Ecdyonurus* sp. was chosen for further experiments.

Previous work on *R. semicolorata* has been scanty. Percival & Whitehead (1929) and Harker (1951, 1953) are the only ones to have made any first-hand observations on its behaviour. Harker (1953) states that the sucker-like arrangement of the gills of *R. semicolorata* gives a greater adhesive power than is found in other British species of mayfly nymphs. Hora (1930), whilst not having any live *Rhithrogena* at hand, offers some views on the sucker-like arrangement of the gills, based mainly on a similar ephemerid nymph *Iron* sp. from India. His 'Venturi' tube theory, however, could not be upheld as by direct observation under a lens *Rhithrogena* was seen to keep all its gills overlapping. Two American species of *Iron* are described by Dodds & Hisaw (1924) who put forward an explanation of the clinging properties of these species. Of the above workers, however, only Harker has made a serious attempt to examine critically the current-resisting properties of *Rhithrogena* in the laboratory.

METHODS AND MATERIALS

Nymphs were collected from the Craigton Burn, Dunbartonshire, and were kept alive in an artificial cascade similar to that used by Hynes (1941).

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In order to examine the current-withstanding powers of the nymphs, special apparatus was designed and constructed (Fig. 1). A reservoir situated in a room above the laboratory maintained a constant head of water of sufficient magnitude. From it water flowed through a rubber hose to a glass tube 1 in. in diameter, 10 in. long and having a 1 × 1 in. side arm almost midway along its length. A Perspex slide, to which strips of Perspex had been cemented to form a cell, was placed midway along this tube, and the water inlet was so arranged that the water flowed directly along the surface of the slide, this surface being the experimental

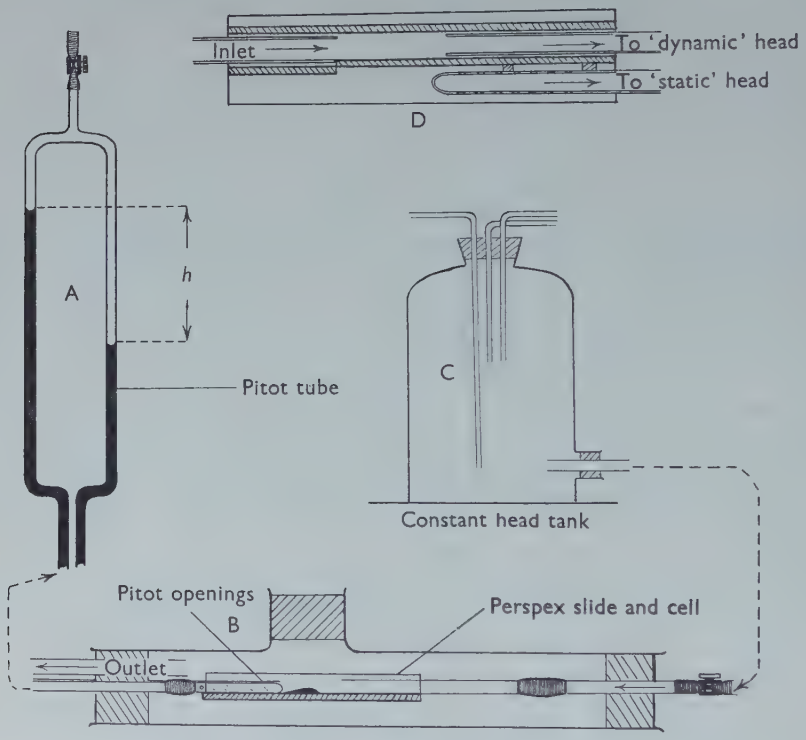

Fig. 1. Diagram of apparatus (not to scale). A, Pitot tube. B, tube containing Perspex cell. C, constant head tank. D, detail plan view of Perspex slide and cell.

substrate. Four such slides were prepared with different surfaces. The tops of two were softened with chloroform, covered evenly with sand, and allowed to dry. Thus, surfaces simulating those found in natural conditions were obtained. Sand passing through a 20-mesh/in. sieve, but stopped by a 30-mesh/in. (i.e. with an average diameter of 0·04 in.) formed surface A, and sand passing through an 80-mesh/in., but stopped by a 90-mesh/in. (approximate diameter being 0·01 in.), formed surface B. One slide was left smooth, surface C, and another was left slightly scratched, the scratches being criss-crossed and approximately 2·0 mm. apart, surface D.

Table 1. *Experimental surfaces*

Surface	Type
A	Sand grains, of average diameter 0·04 in.
B	Sand grains of average diameter 0·01 in.
C	Smooth Perspex
D	Perspex with scratches approximately 2·0 mm. apart

The current was measured by means of a Pitot tube. The opening of the tube to the dynamic head was placed immediately behind the animal so as to obtain as true a reading as possible of the current passing immediately over the nymph; the opening to the static head lay to one side.

The difference in height between the two levels of the Pitot tube was measured initially by means of a reading microscope with a scaled eyepiece, but later a steel rule was used, as the degree of fluctuation of the head (± 2 mm.) did not warrant the more accurate method.

The height in cm. was converted to a current in metres per second by the formula $V = K\sqrt{gh}$, where for the purposes of these experiments K was taken as 1·0, $g = 981$ cm./sec.^{2} and h = difference in height in cm. between the two columns.

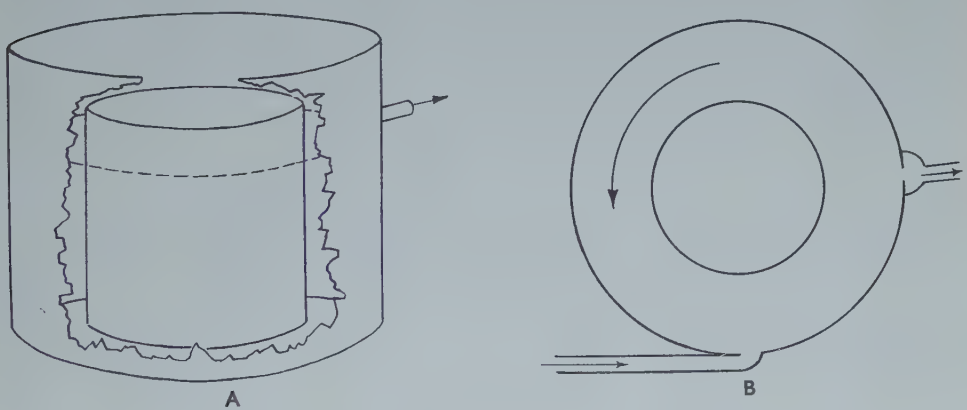

Fig. 2. Adapted print washer. A, cut-away diagram. B, plan view.

In a final experiment use was made of an adapted photographic print-washer (Shelford, 1930, p. 74). This (Fig. 2) was fitted with a central cylinder and so formed an annular trough. The bottom and sides were of pitted metal and enamel, thus providing a surface not quite smooth.

EXPERIMENTAL

The effect of current velocity and degree of roughness of the substrate on *Rhithrogena* and *Ecdyonurus* nymphs has been investigated by means of the above apparatus. Unfortunately, it was impossible in winter to obtain *Rhithrogena* of reasonable size,

and those used initially were of 4·0–5·0 mm. in length. Later, larger nymphs 7·0 mm. long were used.

The experiments were carried out at room temperature 16·7° ± 2·2° C. Each nymph was first examined to make sure that all the gills were intact, measured for length, and then inserted through the side arm on to a prepared slide by means of a camel-hair brush. The animal was restricted by the small cell on the slide, and was compelled to remain there with its head facing the inlet tube. The flow of water was then turned on, increased gradually and as far as possible at a constant rate. At a certain current the animal could no longer maintain a hold on the substrate and was washed off to be collected later. The difference in heights of the Pitot arms was read quickly and noted.

The above procedure could not always be carried out successfully owing to various technical difficulties. Some animals refused to keep themselves orientated with their heads pointing against the flow, others refused to settle and persisted in swimming, while most attempted to avoid the current.

To obtain results in which conditions were as constant as possible, animals which did not face upstream were not included in the calculation of a mean for a specific surface. It was noticed, however, that it did not make a great deal of difference to their holding powers which way they faced. One which faced downstream in Exp. 2 withstood a current of 1·58 m./sec. which compared favourably with most of those which faced upstream. Nymphs which were knocked off the slide by air bubbles originating from airlocks in the water system were also disregarded.

Table 2. *Summary of experiments*

Exp.	Animal under observation	Remarks	Length of nymph (mm.)	No. in sample	Experimental surface	Average difference in heights of Pitot heads in cm. (*h*)	S.D. of (*h*)	Average current in m./sec. at which nymphs lost hold of substrate
1	*R. semicolorata*	—	4·0–5·0	20	B	10·35	1·58	1·43
2	*R. semicolorata*	—	4·0–5·0	20	A	7·15	1·81	1·18
3	*R. semicolorata*	Refused to settle	4·0–5·0	—	C	—	—	—
4A	*R. semicolorata*	—	7·0	5	D	11·98	5·96	1·53
4B	*R. semicolorata*	Clawless; note selection of results	7·0	5	D	4·86	1·96	0·98
5	*Ecdyonurus* sp.	—	6·0–7·5	5	D	12·56	2·53	1·57
6A	*R. semicolorata*	—	6·0–7·0	5	B	15·98	2·62	1·77
6B	*Ecdyonurus* sp.	—	7·0–7·5	5	B	15·42	2·66	1·74
7	*Ecdyonurus* sp.	—	6·0	5	A	7·82	1·63	1·24
8	*R. semicolorata* *Ecdyonurus* sp.	—	Print washer experiment					

In Exp. 1 it was noticed that even at moderate currents the nymphs used their claws rather than their gills for adhesion. The sand grains on this surface B would not be big enough to interfere with the action of a sucker.

Table 3. *'T' test for significance of results*

Comparisons (Exp.)	D.F.	*T*	Significance	*T* 5%
1 and 2	38	5·84	S	1·96
4A and 5	8	0·50	NS	2·31
*4B and 5	8	6·55	S	2·31
6A and 1	23	5·31	S	2·07
6A and 6B	8	0·50	NS	2·31
7 and 6B	8	5·99	S	2·31

NS = not significant. S = significant

The grains of sand on surface A in Exp. 2 were so big that any sucker formed by the gills would be useless. The lower average current required to sweep the nymph off the slide is probably due to the larger grains providing less suitable holds than the finer grains. It should be noted that the claws can maintain a strong grip, even if only a very minute hold is available.

In Exp. 3 readings could not be taken satisfactorily, since the insect refused to remain on the slide if no holds could be obtained for its claws. The claws of several were amputated, and they were again placed on this slide, it being thought that they would use their suckers, being unable to use their claws. They again refused to settle.

Exp. 4A was set up as it had been thought that perhaps the nymphs, as they showed a disinclination to settle on a smooth surface, would prefer a slightly scratched surface to a smooth one, and would so be persuaded to use gills as a sucker. The animals were placed on the slide in the usual manner and watched under a ×8 lens. None of the nymphs used their gills as a sucker though the scratches were not of sufficient depth or number to make a suction device inoperative.

Differences in the figures obtained would seem to depend merely on how the insect was positioned relative to the scratches. One which stayed on till a current of 2·01 m./sec. was reached had all its six claws in good holds, whilst the others had one or more of their claws out of action due to there being no convenient scratches in which to place them.

In Exp. 4B the nymphs' claws were amputated in an attempt to compel the animals to use their gills as suckers. Though these nymphs were eventually persuaded to settle by leaving them in still water for some time, great difficulty was experienced in keeping them in one position. They were easily swept away and few could be persuaded to stay on the slide. So far as could be seen, the nymphs which could be persuaded to settle did use their gills for adhesion. It should be noted that the number of nymphs rejected as unsuitable in this experiment was very much greater than in the other experiments due to the refusal of almost all the nymphs to settle once the claws had been amputated. The readings, however, despite this greater selection, are still smaller than in Exps. 4A and 5.

* The significant difference in this comparison is very much greater than indicated as the animals used in Exp. 4B were more highly selected than those in Exp. 5 and the other experiments (see text).

Since normal *Rhithrogena* did not appear to use its gills as a sucker on the scratched slide (surface D) and persisted in using its claws, it appeared reasonable to predict that *Ecdyonurus* would also be able to maintain a hold under the same conditions. In Exp. 5, therefore, *Ecdyonurus* sp. was placed on the same slide as used in Exp. 4A.

Since *Ecdyonurus* has no suction device it has no alternative other than to use its claws, and as can be seen from the above results it compares favourably with *Rhithrogena*, though the animals used were slightly larger than in the previous experiment. There is no significant difference between the readings obtained in this experiment and in Exp. 4A.

Exps. 6A and 6B were carried out to ascertain whether there was much difference in the current-resisting powers of *Rhithrogena* and *Ecdyonurus* when placed on what would seem to be an optimal surface. There is no significant difference in the holding powers of the animals tested in these experiments though this conclusion should perhaps be qualified by pointing out that the *Ecdyonurus* nymphs used were slightly larger than those of *Rhithrogena*. The sizes of the nymphs do make a difference at least in *Rhithrogena*, since by comparing the figures obtained in Exp. 6A with those obtained in Exp. 1 it can be seen that significantly higher current speeds can be withstood by nymphs of 7·0 mm. than by nymphs of 4·0 or 5·0 mm.

The readings in Exp. 7 were taken in order to provide a comparison with *Ecdyonurus* and *Rhithrogena* in Exps. 6A and 6B. The average figure is significantly lower than in Exp. 6B, and may be again accounted for by considering that the holds available are fewer and more widely spaced.

For Exp. 8 the adapted print washer was used instead of the usual apparatus. Clawless *Rhithrogena* nymphs, 7·0 mm. long, were introduced along with similar sized clawed *Rhithrogena* and *Ecdyonurus* nymphs. It was seen that, even at quite gentle currents, the clawless *Rhithrogena* were unable to settle, whilst the *Ecdyonurus* and *Rhithrogena* with claws quite easily caught hold of the bottom or the sides by means of their claws. This experiment, while not so critical as the previous experiments in that no currents were measured, nevertheless, confirms the previous results more graphically.

DISCUSSION

Various workers at various times have noted the sucker-like arrangement of the gills of *Rhithrogena* and of the similar genus *Iron*. They seem, however, to have placed more emphasis on the gills of *Rhithrogena* as an organ of attachment than is really warranted. The animal appears to use its gills for adhesion only when it finds itself on a surface so smooth that it can get no grip with its claws, or experimentally when its claws have been amputated and it is again on a smooth surface. The gills, however, do not appear to be efficient, and even if there are only minute scratches available as holds the nymphs will use their claws rather than their gills at currents above the most gentle (Exp. 4A).

Percival & Whitehead (1929) have stated that *R. semicolorata* can easily attach

itself to smooth surfaces when the claws are removed. It was found here that while a clawless nymph could be persuaded to settle and use its gills for adhesion when placed on a smooth surface in still water, yet if it was placed in the print washer (Exp. 8) where there was a current it was unable to settle even when the flow of water was comparatively gentle. A similar nymph with claws, when placed in the print washer under similar conditions, obtained holds with its claws on minute scratches in the same manner as did *Ecdyonurus* sp. Percival & Whitehead (1929) have also stated that 'The power of the sucker is very great as it will withstand a powerful jet of water a couple of millimetres away.' As was shown above (Exps. 4A and 4B), however, the figures obtained for animals with claws and animals without claws on a surface which would not render a sucker inoperative indicate that the claws are of greater value to the animal. It should be noted that many nymphs obtained from the stream had one or more gills lost and thus the gills could no longer act as a sucker.

Harker (1951) has carried out a more critical investigation into the current-resisting properties of *R. semicolorata* than Percival & Whitehead (1929). Her results disagree with those obtained in the present experiments in respect of the clinging properties of *Rhithrogena* and *Ecdyonurus*, and in the maximum current required to make a nymph lose hold of the substrate. Harker used a beeswax-lined trough of 56×4 in. and with a constant depth of water of 2 in. She found that 50% of the nymphs of *R. semicolorata* are swept away at a current of 5·2 m./sec. while the greatest current withstood by a *Rhithrogena* nymph of 7 mm. in the present experiments was 2·01 m./sec. The discrepancies in the results are probably due to what is considered a less accurate measurement made by her of the current actually acting on the animal, and to the fact that her nymphs may have been of a different size from the ones in the present experiment. The substrate used, of course, was different and beeswax must provide an excellent, if somewhat unnatural substrate to which nymphs can cling. Apart from absolute discrepancies, however, Harker found that *Rhithrogena* was more successful in resisting currents than either *Ecdyonurus* or *Baetis*. In the stream where the present nymphs were collected, *Baetis* with its torpedo-shaped body always greatly outnumbered *Rhithrogena* and *Ecdyonurus* and appeared to be the most successful of the three genera as was also noted by Dodds & Hisaw (1924) in America. Unfortunately, Harker's paper and thesis were not seen until after the present experiments were completed.

As was shown above, the actual current-withstanding powers of *Ecdyonurus* and *Rhithrogena* are not very different; nor, as will be discussed later, should they be expected to differ.

It was found in the above experiments that the general form of *Rhithrogena* and *Ecdyonurus* could not be described as a current-resisting form, in the same sense as that of *Baetis* which is streamlined and torpedo-like. Hora (1930) considers *Rhithrogena*, *Iron* and other similarly flattened forms to present an ideally streamlined form to the current. He states that the shape (Fig. 3) is streamlined on all sides, and compares it to an aeroplane strut section of what is known as the 'Baby' type (Fig. 3). He does not comment on the fact, however, that the shape of such

nymphs as *Iron* and *Rhithrogena* is really more of an aerofoil, i.e. half a torpedo-shaped form. A body of this shape held stationary in a current in the position shown in the diagram (Fig. 3) experiences a lifting force on the downstream half. This lifting force can be observed in the laboratory, when with increasing current first the body of *Rhithrogena* rises, and then the tail, till the insect finally loses hold and is swept away (Fig. 3). So while the form of *Rhithrogena* is streamlined, this is not considered a current-withstanding adaptation. Dodds & Hisaw (1924) do not mention a dorsal lifting force of the current acting on *Iron* sp. and in fact only mention a lesser downwards force.

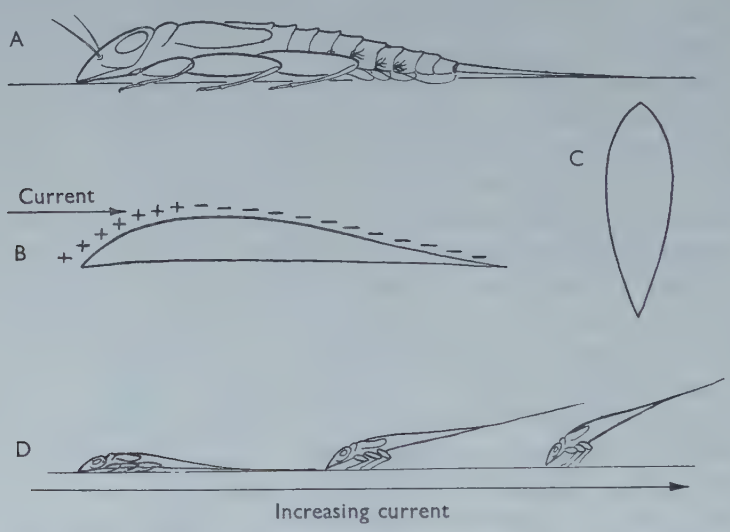

Fig. 3. A, lateral view of nymph of *Rhithrogena semicolorata* (Curtis). B, aerofoil section, + = positive pressure; − = negative pressure. C, 'Baby' strut section. D, diagram showing the result of the lifting effect of increasing current on *Rhithrogena semicolorata* (Curtis).

It was noted on several occasions in the laboratory and in the field that *Rhithrogena* will evade a strong current by sheltering among obstacles and in cracks, rather than face it as is commonly supposed. This, indeed, was one of the main causes of difficulty in forcing nymphs to remain in the current during experiments. If, in the experimental cell, a nymph settled just in front of the water inlet, it was almost impossible to sweep it off the substrate by increasing the current. The thickness of the glass composing the tube was sufficient to shield the nymph from the full force of the current, a current greatly in excess of that normally required to sweep away the nymph.

It is, therefore, postulated here from the above observations and experiments that the form of *Rhithrogena* and *Ecdyonurus* is not designed to withstand currents in the way a torpedo-shaped body does (Hora, 1930; Dodds & Hisaw, 1924), but is designed to enable these animals to utilize cracks and crevices for avoiding the current. It should be noted that in nature the chances are that the currents

meeting the animal will not come strictly in the direction of its long axis (Fig. 3), nor will it be on a perfectly flat surface.

I wish to express my thanks to Dr H. D. Slack, who supervised this work, for much helpful encouragement and criticism.

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My thanks are also due to Prof. E. Percival and to Mr B. B. Given who have both read the manuscript of this paper.

SUMMARY

1. The current-resisting powers of *Rhithrogena semicolorata* Curtis have been investigated and measured, and compared with those of *Ecdyonurus* sp.

2. The part played by the gills as organs of adhesion is considered, and they are found to contribute little to the current-resisting powers of the nymph. The claws are regarded as being of more importance to the animal in clinging than the gills.

3. The dorso-ventral flattening of the animal is considered to be a crevice-seeking adaptation, and not a current-resisting one.

REFERENCES

Dodds, G. S. & Hisaw, F. L. (1924). Ecological studies of aquatic insects. 1. Adaptations of mayfly nymphs to swift streams. *Ecology*, **5**, 140–3.

Harker, J. E. (1951). Unpublished thesis in Manchester University Library.

Harker, J. E. (1953). An investigation of the distribution of the mayfly fauna of a Lancashire stream. *J. Anim. Ecol.* **22**, 1–13.

Hora, S. L. (1930). Ecology, biology and evolution of torrential fauna with reference to organs of attachment. *Phil. Trans.* **218**B, 172–282.

Hynes, H. B. N. (1941). The taxonomy and ecology of the nymphs of British Plecoptera with notes on the adults and eggs. *Trans. R. Ent. Soc. Lond.* **91**, 459–557.

Percival, E. & Whitehead, H. (1929). A quantitative study of the fauna of some types of stream bed. *J. Ecol.* **17**, 282–314.

Rawlinson, R. (1939). Studies on the life history and breeding of *Ecdyonurus venosus* (Ephemeroptera). *Proc. Zool. Soc. Lond.* B, **109**, 377–450.

Shelford, V. E. (1930). *Laboratory and Field Ecology*. Baltimore.

APPENDIX 1

Experiment 1

Rhithrogena of 0·4–0·5 cm. in length on surface B.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·40	11·5	1·50
2	0·40	10·0	1·40
3	0·40	8·6	1·30
4	0·40	12·4	1·56
5	0·40	10·3	1·42
6	0·40	10·5	1·44
7	0·40	8·0	1·25
8	0·40	10·4	1·43
9	0·40	8·9	1·32
10	0·45	7·7	1·23
11	0·40	9·0	1·33
12	0·50	12·1	1·54
13	0·50	13·3	1·62
14	0·40	8·7	1·31
15	0·50	12·0	1·53
16	0·50	10·2	1·42
17	0·50	10·6	1·44
18	0·50	12·5	1·57
19	0·50	10·6	1·44
20	0·40	9·6	1·37

Approximate mean, 1·43 m./sec.

APPENDIX 2

Experiment 2

Rhithrogena of 0·4–0·5 cm. in length on surface A.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·45	4·9	0·98
2	0·40	7·7	1·23
3	0·45	6·8	1·15
4	0·45	6·7	1·15
5	0·40	6·6	1·14
6	0·55	6·8	1·15
7	0·60	10·0	1·40
8	0·45	6·0	1·08
9	0·50	8·4	1·28
10	0·45	5·5	1·04
11	0·40	5·8	1·07
12	0·50	4·1	0·90
13	0·45	9·8	1·39
14	0·40	8·7	1·30
15	0·50	7·8	1·24
16	0·40	9·4	1·36
17	0·40	9·3	1·35
18	0·40	5·4	1·03
19	0·40	4·7	0·96
20	0·40	8·5	1·29

Approximate mean, 1·18 m./sec.

APPENDIX 3

Experiment 4A

Rhithrogena of length 0·70 cm. on surface D.

	Size—length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·70	6·5	1·13
2	0·70	6·5	1·13
3	0·70	20·5	2·01
4	0·70	14·3	1·67
5	0·70	12·1	1·54

Approximate mean, 1·53 m./sec.

APPENDIX 4

Experiment 4B

Rhithrogena of 0·70 cm. length, without claws on surface D.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·70	8·1	1·26
2	0·70	4·5	0·93
3	0·60	3·0	0·77
4	0·70	3·8	0·86
5	0·70	4·9	0·98

Approximate mean = 0·98 m./sec.

APPENDIX 5

Experiment 5

Ecdyonurus 0·6–0·75 cm. on surface D.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·75	14·4	1·68
2	0·75	14·9	1·71
3	0·60	13·5	1·63
4	0·70	8·9	1·32
5	0·75	11·1	1·48

Approximate mean = 1·57 m./sec.

APPENDIX 6

Experiment 6A

Rhithrogena 0·6–0·7 cm. on surface B.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·60	14·7	1·70
2	0·70	14·6	1·69
3	0·70	16·2	1·78
4	0·70	20·5	2·01
5	0·75	13·9	1·65

Mean = 1·77 m./sec.

APPENDIX 7

Experiment 6B

Ecdyonurus 0·6–0·75 cm. on surface B.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·75	16·3	1·79
2	0·60	14·7	1·70
3	0·75	13·0	1·60
4	0·75	18·5	1·91
5	0·75	14·6	1·61

Mean = 1·74 m./sec.

APPENDIX 8

Experiment 7

Ecdyonurus of 0·6 cm. on surface A.

	Length (cm.)	Pitot head (cm.)	Velocity of current (m./sec.) ($V=\sqrt{2gh}$)
1	0·60	8·1	1·26
2	0·60	9·3	1·35
3	0·60	6·4	1·12
4	0·60	5·9	1·08
5	0·60	9·4	1·36

Mean = 1·24 m./sec.

THE OCCURRENCE OF 5-HYDROXYTRYPTAMINE IN SCORPION VENOM

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(With Plate 1)

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The constituents of scorpion venom have not yet been fully identified. During preliminary investigation of the venom of *Leiurus quinquestriatus* by paper chromatography, a spot corresponding in position to that due to 5-hydroxytryptamine (HT) was seen. This paper is concerned with the methods of identification and assay of this substance in venom, and also considers the histochemistry of the venom gland in relation to this finding.

METHODS

Venom was obtained exclusively from *Leiurus quinquestriatus* (Hemprich & Ehrenberg, 1829). It was collected by stimulating the telson, using an induction coil. The pooled venom was dried and kept in a vacuum desiccator. Its toxic potency did not appear to vary over a period of several weeks. It was redissolved in water as required.

Ascending paper chromatography was carried out using Whatman papers nos. 1 and 4, and the following solvent mixtures: *n*-butanol, acetic acid, water (4:1:5); amyl alcohol, pyridine, water (2:2:1); *n*-butanol saturated with N-HCl. Various reagents were used to demonstrate the HT spots, including *p*-dimethylaminobenzaldehyde, diazotized *p*-nitroaniline, and α-nitroso-β-naphthol. The green-blue colour in ultra-violet light after treatment with an acetic acid-ninhydrin mixture (Jepson & Stevens, 1953) was probably the most useful and sensitive method. It also demonstrated other venom constituents by virtue of the ninhydrin reaction. Paper electrophoresis was also used, and good resolution of the constituents was obtained in normal acetic acid (pH 2·3).

The assay of HT was mainly carried out on the isolated rat uterus (by the method of Amin, Crawford & Gaddum, 1954), but estimations were also made on the isolated guinea-pig ileum. For fluorometric estimations, the venom was extracted with 90% (v/v) acetone. This extract was dried, redissolved in 3N-HCl, and the HT estimated in a Farrand spectrofluorometer, using an activating wave-length of 300 mμ and measuring the fluorescence at 540 mμ (cf. Bogdanski, Pletscher, Brodie & Udenfriend, 1956). In all cases, the HT used as the standard was the creatinine sulphate complex, but all quantities are expressed as the base.

For histological examination, venom glands were dissected free of chitin, fixed in buffered 10% formalin (with the addition, for the chromaffin reaction, of 3% potassium dichromate), and embedded in paraffin. The argentaffin reaction was obtained with Fontana's ammoniacal silver solution as detailed by Gomori (1952). The diazo reagents for demonstrating enterochromaffin granules were: diazotized *o*-safranin (Lillie, Burtner & Henson, 1953), fast red salt B, and diazotized *p*-nitroaniline (Pearse, 1953). Ehrlich's reaction with *p*-dimethylaminobenzaldehyde was also carried out by the method of Pearse (1953).

RESULTS

Paper chromatography. 0·5 mg. quantities of venom were run simultaneously with standard HT, and, in all three solvent mixtures, distinct spots with the same flow rates and colour reactions as the standard HT were obtained. The sizes of the spots, compared with known quantities of pure HT, indicated concentrations of about 1–5 μg. HT/mg. dry weight of venom. No other spots appeared after treatment with the above colour reagents, except in the case of ninhydrin which showed other components with lower rates of flow and poorly separated. Better resolution of these components was obtained by paper electrophoresis, and here too a line was found having the same mobility as pure HT.

Assay of HT in venom. Various batches of crude venom were assayed on the isolated rat uterus, and were found to contain 2–4 μg. HT/mg. dry venom. This oxytocic activity could be abolished completely by dihydroergotamine, a potent antagonist of HT (Gaddum & Hameed, 1954). A similar result was obtained with assays on isolated guinea-pig ileum, though this method is less precise. One batch of venom was extracted with acetone, and the extract assayed fluorometrically. In this case the concentration of HT was found to be 2·9 μg./mg. venom. The fluorescence spectrum of the extract appeared identical with that of pure HT.

Histology of the venom gland. The gland has two lobes, the lumen of each communicating with the sting. Each lobe consists of folded epithelium with rather sparse supporting cells surrounded by a coat of smooth muscle. After formalin fixation, the epithelium is highly granular, and a considerable proportion of these granules are capable of reducing ammoniacal silver nitrate (Pl. 1, fig. 1). They also show to some extent a chromaffin reaction after fixation in a formalin-dichromate mixture. However, the diazo methods, which demonstrate mammalian enterochromaffin granules clearly, gave negative results, and no fluorescence was seen when sections were viewed in ultra-violet light. Some cells in the basal parts of the gland gave a clearly positive indole reaction with *p*-dimethylaminobenzaldehyde (Pl. 1, fig. 2). Usually two or three of such cells could be seen in each section.

DISCUSSION

The ubiquity of HT has been noted before (Erspamer, 1954), and it had already been found in wasp venom (Jaques & Schachter, 1954) when the present results were obtained. Its concentration in the venom of *Leiurus quinquestriatus* is

apparently the highest yet reported in any biological medium, but there is no indication as yet of any function it may perform. The considerable local pain resulting from a sting may be due to the HT content of the venom (cf. Armstrong, Dry, Keele & Markham, 1952). This point may be settled by experiments using venom from which the HT has been extracted. It is perhaps relevant that Mohammed & El Karemi (1953) have claimed that atropine and dihydroergotamine, both of which can antagonize HT, can protect rats to some extent against injected scorpion venom.

The high concentration of HT in the venom adds interest to the histochemistry of the gland. From the results given above, it would appear that there are no cells present which closely resemble the mammalian enterochromaffin cell. Although some cells show argentaffin and chromaffin granules, they do not give the diazo reactions or fluoresce in ultra-violet light, and the granules themselves are generally much coarser than those seen in the enterochromaffin cell.

The indole reaction given by some basal cells may mean that these are the producers of HT. Enterochromaffin cells do not give the indole reaction, and Barter & Pearse (1955) have suggested that, after formalin fixation, HT is converted by ring closure into a β-carboline derivative. In the present case, however, it does not seem likely that this reaction occurred since no fluorescence in ultra-violet light was seen, and it remains possible that the indole reaction does indicate the site of HT.

SUMMARY

5-hydroxytryptamine has been identified as one of the constituents of the venom of *Leiurus quinquestriatus* and its concentration estimated to be 2–4 μg./mg. dry weight of venom. The relevant histochemistry of the venom gland is discussed.

Our thanks are due to Prof. D. A. Smith for his advice and encouragement; to Dr T. B. B. Crawford, Pharmacology Department, University of Edinburgh, for the spectrofluorometric estimation; and to Abbott Laboratories, Chicago, for a gift of 5-hydroxytryptamine creatinine sulphate.

REFERENCES

Amin, A. H., Crawford, T. B. B. & Gaddum, J. H. (1954). The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. *J. Physiol.* **126**, 596–618.

Armstrong, D., Dry, R. L. M., Keele, C. A. & Markham, J. W. (1952). Pain-producing actions of tryptamine and 5-hydroxytryptamine. *J. Physiol.* **117**, 70–1*P*.

Barter, R. & Pearse, A. G. E. (1955). Mammalian enterochromaffin cells as the source of serotonin. *J. Path. Bact.* **69**, 25–31.

Bogdanski, D. F., Pletscher, A., Brodie, B. B. & Udenfriend, S. (1956). Identification and assay of serotonin in brain. *J. Pharmacol.* **117**, 82–8.

Erspamer, V. (1954). The pharmacology of indolalkylamines. *Pharmacol. Rev.* **6**, 425–87.

Gaddum, J. H. & Hameed, K. A. (1954). Drugs which antagonize 5-hydroxytryptamine. *Brit. J. Pharmacol.* **9**, 240–8.

Gomori, G. (1952). *Microscopic Histochemistry*. Chicago: University of Chicago Press.

Jaques, R. & Schachter, M. (1954). The presence of histamine, 5-hydroxytryptamine and a potent slow-contracting substance in wasp venom. *Brit. J. Pharmacol.* **9**, 53–8.

Jepson, J. B. & Stevens, B. J. (1953). A fluorescence test for serotonin and other tryptamines. *Nature, Lond.*, **172**, 772.

Lillie, R. D., Burtner, H. J. & Henson, J. P. G. (1953). Diazo-safranin for staining enterochromaffin. *J. Histochem. Cytochem.* **1**, 154–9.

Mohammed, A. H. & El Karemi, M. (1953). An antidote to scorpion toxin. *J. trop. Med.* (*Hyg.* **56**, 58–9.

Pearse, A. G. E. (1953). *Histochemistry*. London: J. and A. Churchill, Ltd.

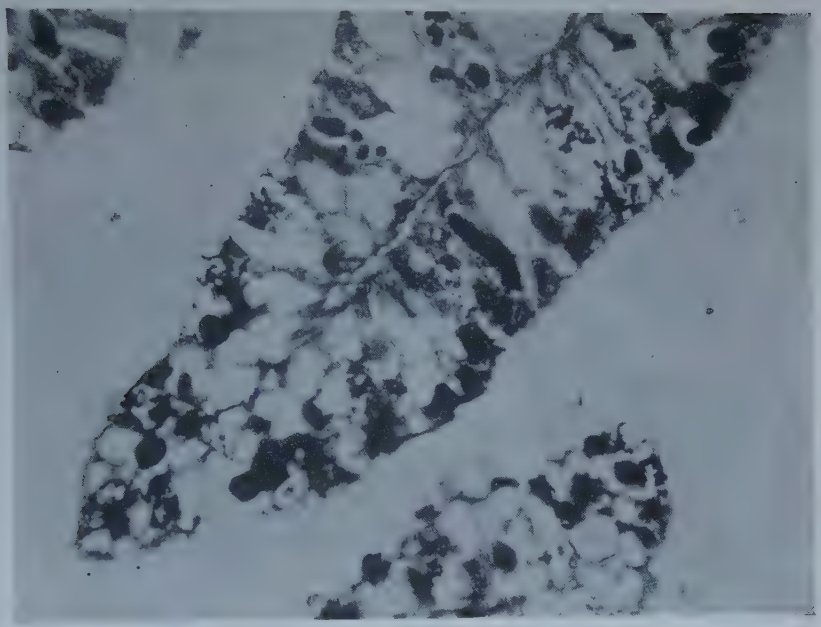

Fig. 1. Epithelium of venom gland. Formalin fixation. Argentaffin reaction. ×200.

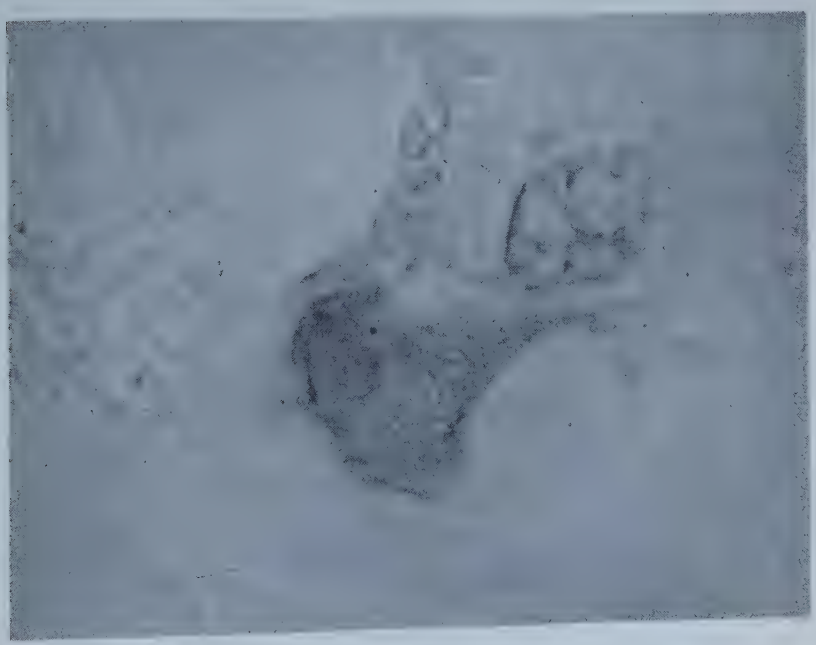

Fig. 2. Cell from basal part of gland showing Ehrlich (indole) reaction. ×760.

SEASONAL AND EXPERIMENTAL VARIATIONS OF THE OXYGEN CONSUMPTION OF THE LIMPET *ANCYLUS FLUVIATILIS* (O. F. MÜLLER)

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INTRODUCTION

A seasonal variation of the respiration of the limpet family Ancylidae was found by the senior author (Berg, 1952) during earlier studies on the oxygen consumption, but its character was not understood. It was emphasized 'that further experiments have to be carried out to test the seasonal variation of the respiration—its magnitude, and the possible correlation between reproduction period and oxygen consumption...'. The main purpose of the studies reported now is to elucidate this problem. To this end the oxygen consumption of the species chosen for the investigation, *Ancylus fluviatilis*, was determined at various seasons and the results compared.

To compare the oxygen consumption at various seasons we must know the accuracy of the determinations, and the influence on the results of the way in which the experiments are carried out and the animals treated. The latter is important, owing to the fact that the rate of respiration of poikilothermic animals is so easily influenced by experimental conditions.

An investigation of the respiration of poikilothermic animals for *ecological* reasons always involves comparison between results from various localities, and at various seasons; comparison of results concerning successive generations of animals, different geographical races or different species, and so on. Mostly the respiratory differences studied cannot be expected to be very great, and for that very reason the influence of various experimental conditions on the rate of respiration must be investigated.

Furthermore, it must be remembered that warm-blooded animals at complete rest in a starving condition are characterized by a definite level of metabolism, the so-called standard or basal metabolism. But 'it is important to note that in many cold-blooded animals there is no such well-defined basal level' (Krogh, 1941; cf. Zeuthen, 1947). For this reason, too, it is of interest to check the observed results on poikilotherms by controlled experiments capable of showing the influence of various experimental conditions.

II. METHODS

(*a*) *The oxygen determinations*

The determinations were made by a polarometric method developed by Bartels (1949, 1950) and used earlier for a similar purpose by Berg (1953). The apparatus

consists of two electrodes, one a dropping mercury electrode and the other a calomel electrode. A small voltage is applied between them using a 2·4 V. battery, and the resulting current is measured by means of a sensitive galvanometer. Under certain circumstances the deflexions of the galvanometer are proportional to the oxygen pressure in the water of the analysis bottle.

In order to get rid of undesired maximal deflexions of the galvanometer, it is recommended to add a suppressing agent, an organic substance, to the water of the analysis bottle. In the first experiments three drops of a 1·25% gelatine solution were added before measurements were made. But as it turned out that the power of suppression of the gelatine solution was not constant, but decreased rapidly during the experiments, sometimes during 1 hr., a solution (0·5%) of tylose (methylcellulose) in distilled water was used. This agent has the advantage that its power of suppression is preserved unchanged for several days.

Before a measurement of the oxygen content of water can be carried out a calibration curve (a straight line showing the relation between known values of oxygen pressure in water and deflexions of the galvanometer) must be constructed experimentally. Later on by means of this calibration curve unknown oxygen contents corresponding to observed deflexions of the galvanometer are determined.

The method has the advantage that it is possible to use respiration bottles containing only 4–6 ml. of water and is therefore suitable for small numbers of experimental animals. When everything is prepared in advance many measurements can be carried out in a short time, but it is a disadvantage that the method cannot be used directly in all natural waters without tylose or other substances being added.

(*b*) *Notes on the experiments*

Experiments in which the limpets were used only once. Where not otherwise indicated the experiments were carried out as follows. The animals were collected in the stream, Funder Aa, or in the littoral zone of the eutrophic lake, Rørbaek Sø, Jutland, put in a Dewar vessel with water from the locality and brought to the laboratory. The journey lasted *c.* ½–1 hr. The animals were then placed in aerated water in a thermostatic bath. The usual variation of the temperature of the thermostat was ±0·1° C.

The experiments were made in closed respiration chambers, that is, in bottles containing 4–6 ml. of aerated water, the volumes of which were known exactly. The oxygen content of the water was measured before the experiment. During the experiment the bottles were placed in darkness in the thermostat. The duration of an experiment was 1 hr. Apparently, the rather sluggish animals usually did not move much during the experiment; no narcotization was used. After the experiment the oxygen content of the water was measured directly in the respiration bottle. At the end of the experiments the oxygen content of the bottles was usually 60–70% of the air-saturation value. Earlier experiments (Berg, 1952) have shown that such a decrease does not perceptibly influence the oxygen consumption of *A. fluviatilis*. The difference between the two oxygen determinations is the oxygen consumed. The limpets were dried on filter paper, weighed, and killed in boiling water; the

limpet shells were then dried and weighed. The difference between the first and the last weighing is the weight of the tissues or the live weight of the animals. It is important to standardize the technique of drying on filter paper, otherwise significant errors occur.

The water used in the respiration bottle was taken from a nearby lake, Borresø. A test was made to determine whether a decrease in the oxygen content of the lake water took place during 1 hr. when there were no animals in the closed respiration bottles. A series of five experiments with water aerated with atmospheric air, and another series equilibrated with a mixture of 16·4% oxygen in nitrogen were carried out. It was found that in both cases no decrease of the oxygen content took place.

The oxygen consumption found in experiments was calculated in μl. per individual of a particular live weight (in mg.) per hour. This is better than calculating the oxygen consumption per gramme, as such a figure varies according to the size of the animal, large ones usually having a lower oxygen consumption per gram than smaller ones.

The size of the individuals of *A. fluviatilis* varies considerably during a year. In order to make the oxygen consumption per individual found at a certain time comparable with results from other seasons, the oxygen consumption was always calculated per individual with a live weight of 20 mg. This was done in the following way.

Every experiment on the oxygen consumption of an individual of 20 mg. live weight was based upon a series of five (or in some cases ten) separate determinations of the type described above. The animals selected for the series of determinations might vary from 5 to 40 mg. live weight (and sometimes even more), but all individuals placed in the same respiration bottle were carefully selected to be of the same size. About seven to eight small, or four to five middle-sized, or one to two large limpets were put into any respiration bottle. Thus, twenty-five to thirty specimens were used in every series of experiments.

The results of each series of five (or ten) determinations were plotted on a graph as in Fig. 1, where the oxygen consumption per individual per hour is plotted against the mean live weight of the individuals, both on a logarithmic scale. The relation of oxygen consumption to weight plotted in this way is a straight line (cf. for example, Berg, 1952, 1953). Since it was found from the first series of determinations that the slope of the straight line seemed to vary about 0·73 (cf. Hemmingsen, 1950) this slope was used in all experiments reported in this paper. (Later on a test was made to discover how much the slope of the line relating oxygen consumption to weight varied, cf. p. 66.) From the figure the oxygen consumption of a *standard individual* having the live weight of 20 mg. was read off.

The reason for choosing the value 20 mg. as the live weight of the animals to be compared is that this figure is about the mean weight of the species. Therefore, it generally has the smallest standard error in a series of determinations.

It will thus be noted that the determination of the rate of respiration at a given moment is based on several experiments involving many individuals.

Experiments in which the limpets were used several times. In the method described

above the determination of oxygen after the experiment is carried out directly in the respiration bottle, and the drops of mercury fall into the water of the respiration bottle while it still contains the experimental animals. Such animals are poisoned by the mercury and cannot be used for further experiments. However, in certain experiments it was desirable to use the same animals several times, so a test was made to discover whether a significant uptake of oxygen occurred when the water from the respiration bottle was decanted to another bottle before measurements were made, thus enabling the limpets to escape poisoning. The test was carried out as follows.

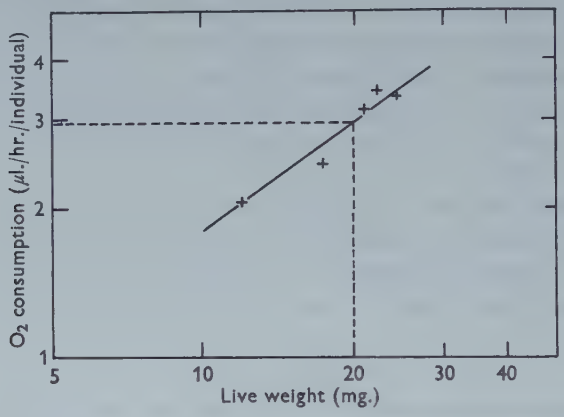

Fig. 1. Diagram showing the graphical method of calculating the oxygen consumption of *A. fluviatilis* of the standard live weight 20 mg. The slope of the line relating oxygen consumption to live weight is 0·73. For explanation see text.

In a thermostat at a temperature of 16° C. a large bottle containing filtered water from the lake Borresø was equilibrated for at least 10 min. with a mixture of 11·9% oxygen in nitrogen. A 6 ml. respiration bottle of the type used in all our experiments was then washed and filled with the equilibrated water. Part of this water was afterwards poured carefully into a *c.* 4 ml. bottle along its inner side, reduced by means of the stopper to a certain volume, and three drops of a 1·25% gelatine solution were added to it. Finally, the oxygen content was determined polarometrically. After five measurements, five others were carried out without any decantation of the water. The results of the two series of measurements are given in Table 1.

Table 1

I. With decantation deflexion of the galvanometer (mean)	II. Without decantation deflexion of the galvanometer (mean)
53·9	53·2
53·65	53·45
53·5	53·6
53·7	53·5
53·3	54·0
Average 53·81	Average 53·55

The result shows that the oxygen uptake during one decantation is very small. The error caused thereby is less than 1%, when the deflexions of the galvanometer are reduced from 90 to 60, which is a normal case.

Hence, the error resulting from a single decantation can be neglected. The advantage is that the animals avoid the mercury and its poisoning influence. They can therefore be used again for fresh experiments.

(*c*) *How great an influence has the volume of the experimental animals on the accuracy of the determination of their oxygen consumption?*

The volume of the experimental animals is not taken into account in the manner of calculating the oxygen consumption. The volume of the respiration bottles is regarded as exact, and the smaller or greater volume of water which the experimental animals displace is disregarded. This is not quite correct; a systematic error is introduced in this way and its magnitude is dependent on the volume of the animals. The error must be greater at low than at high experimental temperatures, since a greater number of animals is used in the first case than in the latter in order to obtain an appropriate decrease of oxygen in the respiration bottle.

How great this error may be was tested in two experiments carried out at 3° C. in February 1954. The volume of the experimental animals was found, deducted from the volume of the respiration bottles and the oxygen consumption computed on the basis of the net volume of the water in the respiration bottles. In this way it turned out that the oxygen consumption computed in the ordinary way was 7·7% (at 3° C.) and 3·8% (at 11° C.) higher than the corrected values. At higher temperatures the error must be still less. It deserves notice that most of the experiments in this paper were carried out at 11° C. or at higher temperatures.

When one takes into account the fact that the tissues of the animals contain oxygen, the figures calculated above will be reduced. Also, when one is comparing the oxygen consumption in two experiments the error will be approximately the same in both, and may therefore be disregarded.

(*d*) *Accuracy of measurements*

By means of the graphical method shown in Fig. 1 (p. 46) the respiration of an individual weighing 20 mg. is found; the question then arises how reliable such a determination is. How great is the standard deviation for the average respiration of individuals of 20 mg.? The answer to this question has been given by two different statistical calculations, as follows.

(1) On the basis of three series of experiments including altogether twenty-two experiments (that is 110 determinations of the O_2 consumption), the common regression equation was calculated, assuming a slope of 0·73, as

$$\log y = a + b \log x = -0{\cdot}3993 + 0{\cdot}73 \log x.$$

If the weight of an animal of 20 mg. is designated $x_{20\,\mathrm{mg.}}$, and the associated respiration $y_{20\,\mathrm{mg.}}$, the standard deviation for $\log y_{20\,\mathrm{mg.}}$ is found to be $s = 0{\cdot}0081$, and $\log y_{20\,\mathrm{mg.}} = 0{\cdot}5504$. Thus, in this case the respiration of an animal weighing 20 mg. averages 3·55 μl. The standard deviation for this respiration value is 1·85%; and

the 2·5% fiducial limits ($\log y = 0{\cdot}55 \pm 1{\cdot}96s$) are 3·42 and 3·68 μl., i.e. ±3·65%. This means that the standard deviation for $y_{20\,mg.}$ is 2%, and there is a 95% probability that a new respiration experiment of the same type as the twenty-two experiments mentioned above would give a result which would at most deviate *c.* ±4% from the average of the twenty-two experiments.

(2) From a single series of experiments including five experiments (which means twenty-five determinations of the O_2 consumption) of the type of which the result is shown in Fig. 1, the standard deviation for the respiration $y_{20\,mg.}$ (cf. Hald, 1948) was calculated. This series was chosen for calculation because the experiments had a large correlation coefficient, $r = 0{\cdot}99$. The calculated regression equation for these experiments was $\log y = -0{\cdot}6133 + 0{\cdot}7439 \log x$. The standard deviation for $\log y_{20\,mg.}$ was $= 0{\cdot}0094$, after which was found $\log y = 0{\cdot}3545$. Therefore, the respiration of an animal of 20 mg. was 2·26 μl. The standard deviation of this mean is calculated as 2·2%, and 2·5% fiducial limits ($\log y = 0{\cdot}354 \pm 1{\cdot}96s$) gave the result 2·17 and 2·36 μl., i.e. ±4·2%.

The latter calculation of standard deviation and 2·5% fiducial limits are based on a smaller sample than those calculated under (1), and as already mentioned, the slope of the regression line is in this case calculated. But actually the results of the two modes of calculating agree well with each other.

Even though all the statements of oxygen consumption in this paper are based on five (or in some cases ten) determinations, as explained by means of Fig. 1, it would also be of interest to know how safely a single one of these determinations can give the rate of the respiration. In order to answer this question a calculation was made of the standard deviation and the 2·5% fiducial limits (corresponding to $\pm 1{\cdot}96s$) for four experiments of the type described in Fig. 1, but each comprising ten separate determinations. It was assumed that the inclination of the regression line was 0·73 (see p. 45). Stating standard deviation and 2·5% fiducial limits in percentage of the mean, results are given in Table 2.

Table 2

Exp.	Standard deviation	2·5% fiducial limits
1	*c.* 7% of the mean	*c.* 13% of the mean
2	*c.* 10% of the mean	*c.* 20% of the mean
3	*c.* 12% of the mean	*c.* 23% of the mean
4	*c.* 15% of the mean	*c.* 30% of the mean

On the average one can reckon with a standard deviation of *c.* 11% and a 2·5% fiducial limit of *c.* 22% of the mean.

The standard deviation mentioned above, 11%, is of course appreciably greater than the one caused by the method of measurement. Earlier it has been shown (Berg, 1953) that the calibration curve, which must be determined by the polarometric method showing the relation between the oxygen content of water and the galvanometer deflexion, has standard deviations of *c.* 2% for the values of oxygen measured. In measuring the oxygen consumption two oxygen measurements are

used, one before and one after the consumption. On the basis of the standard deviation found earlier, 2%, and assuming the theoretical oxygen consumption to be of the magnitude usually found in the experiments with *A. fluviatilis*, the standard deviation of the oxygen consumption in percentage of the theoretical oxygen consumption has been calculated at 5·8%. This standard deviation of *c.* 6% only indicates the uncertainty due to the method of measurement.

The total standard deviation, that is, the uncertainty caused by the method of measurement method, plus the uncertainty deriving from the animals, is found on the basis of the four above-mentioned experiments for the consumption of oxygen to be *c.* 11%. This is—as might be expected—essentially greater than the standard deviation, 6%, which has been calculated solely by taking into consideration the uncertainty of the calibration curve.

The relation between the total standard deviation, s_t, the one resulting from the method, s_m, and the physiological one, s_f, is $s_t^2 = s_m^2 + s_f^2$. As $s_t = 11\%$ and $s_m = 6\%$, $s = 9{\cdot}2\%$. Thus, the standard deviation due to differences between the limpets is *c.* 9%; it is seen that the cause of the variation of the single determination is essentially of a physiological nature.

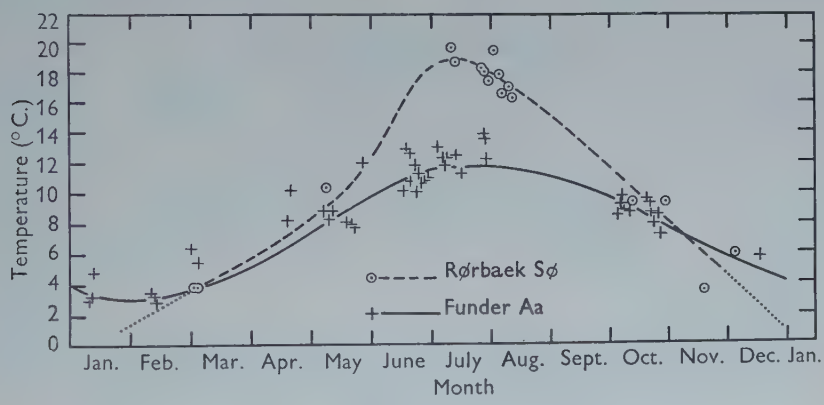

Fig. 2. Temperatures at various seasons in two localities of *A. fluviatilis*, the littoral zone of Rørbaek Sø and the river Funder Aa, 1951–55.

III. SEASONAL VARIATION IN THE OXYGEN CONSUMPTION

The limpets used for experiments were collected in two localities in Jutland with fairly different temperatures during the year, namely, the river Funder Aa and the lake Rørbaek Sø. Funder Aa is a small river, *c.* 4–5 m. wide, almost a brook. It has a strong current, receives much spring and ground water, and has a very constant flow of water throughout the year (cf. Danske Hedeselskab, 1940). As it receives much spring and ground water its temperatures in the summer are low, mostly about 11–12° C. (Fig. 2). During very still, hot summer days the temperature of the river may be 14–16° C., but this is exceptional, mostly of short duration, and such figures are therefore not included in Fig. 2. In winter the river is never covered with ice.

Rørbaek Sø has summer temperatures in the littoral zone varying from *c.* 16 to 20° C. In the collecting places in the lake there is a slight inflow of spring and ground water and part is shaded by alder; so the summer temperatures are not quite as high as farther out in the lake. In winter the lake is covered with ice at least for a few weeks, often longer.

The seasonal variation of the rate of respiration of *A. fluviatilis* was studied by means of six series of experiments carried out in various ways.

(*a*) *Experiments carried out at temperatures varying during the year, but usually differing by less than* 0·5° C. *from the stream temperature found at the time of collection in the river Funder Aa.* The difference between the experimental and the stream temperature was in no case greater than the temperature variation in the stream as between day and night. The rates of respiration found must therefore be regarded as very similar to the actual respiration of the animals in the stream during the year. For three successive years experiments (each comprising ten determinations) were carried out. Their results are shown in Fig. 3. It will be seen that during the year the respiration varies from 0·61 (February at 3·0° C.) to 3·44 μl. O_2/hr./individual of 20 mg. (end of July at 13° C.). The rate of respiration may thus be about five times as great in summer as in winter.

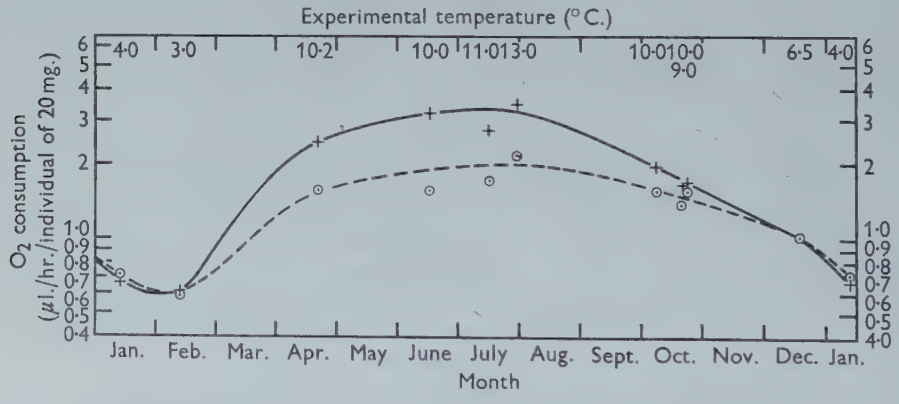

Fig. 3. Oxygen consumption of *A. fluviatilis* from Funder Aa at various seasons (+———+) determined 2 hr. or less after collection of the animals, and at the various temperatures of the river. The experimental temperatures are indicated above. Hypothetical oxygen consumption (⊙- - - - - ⊙) based on the determination from December and computed in accordance with the so-called Krogh's curve. For further explanation see text.

The dotted curve shows what the oxygen consumption would be during the year if the respiration varied according to Krogh's curve (1914, 1916). The measurements from December are used as a basis for the computation of this hypothetical curve, and the other values of the curve are computed by taking into consideration the various deviations of the experimental temperatures from the December temperature, 6·5° C. It will be seen that (1) in winter the continuous curve showing the rate of respiration as determined seems to follow the dotted curve computed according to Krogh; but (2) in other seasons, especially in early summer, it is placed

distinctly higher. It will also be noted that (3) the observed oxygen consumption of the animals is greater in early summer than in autumn at the same temperature (10° C.).

The conclusion drawn from the above must be that the rate of respiration of the limpets from Funder Aa is not adapted to high summer temperatures, i.e. is not lower than should be expected according to Krogh's curve but on the contrary is higher. Furthermore, it is very probable that the high rate of respiration found from April to the end of July is caused by reproductive activity, since the period of reproduction occurs just at that time (see p. 55).

The conclusion drawn of course implies that Krogh's curve—or a very similar relation between oxygen consumption and temperature—is valid in this case (see p. 62).

(*b*) *Experiments carried out at the same temperature during all seasons of the year.* Two series of experiments were made, one at 18° C. and the other at 11° C.

The animals were collected in the river Funder Aa; a short time after collection they were placed in a thermostat in the laboratory at 18 or 11° C. Experiments of the usual type (Fig. 1) were carried out in the period 2–25 hr. after collection and the results are shown in Fig. 4.

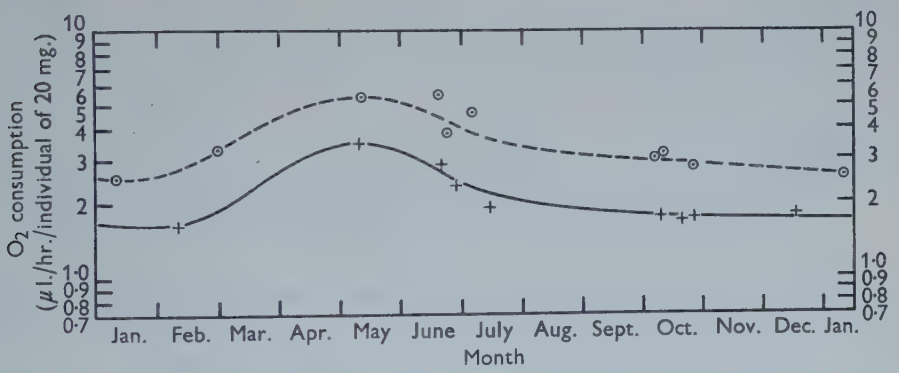

Fig. 4. Seasonal variation of oxygen consumption of *A. fluviatilis* from Funder Aa determined 2–25 hr. after collection of the animals at two constant experimental temperatures, 18° C. (⊙ - - - - - ⊙) and 11° C. (+———+).

As a general rule several experiments were carried out at various times during this period; and in such cases only the mean values are indicated.

The two curves in Fig. 4 show the same course. It is seen that the oxygen consumption is not the same at all seasons, although it is measured at the same experimental temperature throughout the whole of the year. The rate of respiration is found to be distinctly higher in early summer; and the early summer maximum of oxygen consumption is about twice that of the consumption found in autumn and winter at the same experimental temperature.

The oxygen consumption found at 11° C. during the summer maximum agrees well with the oxygen consumption found at about the same experimental tempera-

tures and illustrated in Fig. 3. The periods of the maxima in Figs. 3 and 4 also agree fairly well.

The curve of oxygen consumption found at 18° C. is placed at a level 1·6 times higher than that found at 11° C. Thus, the two curves are placed somewhat nearer one another than they would be if the oxygen consumption had varied according to Krogh's curve. (In this case the values of the upper curve would have been about twice as high as those of the lower one.)

The strange variation of the rate of respiration at the end of the summer is most probably due to variation in the time of completion of the reproductive period from year to year; for, as mentioned above, the results given are combined from three successive years.

(*c*) *Experiments carried out at the same temperature*, 18° *or* 11° C., *during all seasons of the year, as in the above-mentioned case* (*b*), *but measurements made* c. 30–75 *hr. after collection of the animals in the river Funder Aa.* The results from two successive years are combined and shown in Fig. 5.

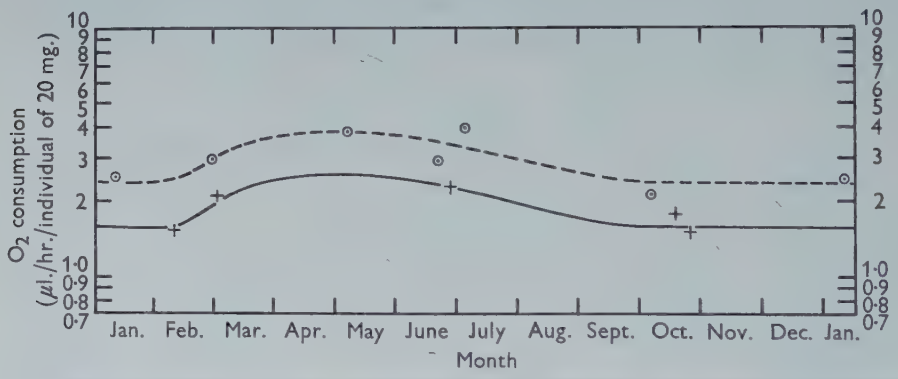

Fig. 5. Seasonal variation of oxygen consumption of *A. fluviatilis* from Funder Aa determined *c.* 30–75 hr. after collection of the animals at two constant experimental temperatures, 18° C. (⊙ - - - - - - ⊙) and 11° C. (+———+).

The curves in Fig. 5 show the same seasonal variation of respiration as the curves in Fig. 4. The maximum during the early summer is, however, not so distinct as in Fig. 4, and both curves in Fig. 5 indicate a respiration reduced a little in comparison with the above-mentioned curves. The explanation is that a starvation due to the experimental conditions (cf. also p. 56) reduced the results a little in the experiments discussed here, and especially made itself felt when the animals had already a fairly high rate of respiration, i.e. in early summer.

(*d*) *Experiments carried out with limpets from the shallow littoral zone of a lake, Rørbaek Sø, at experimental temperatures varying during the seasons, but deviating at most* 1·1° C. *from the temperatures found in the littoral zone at the time of collection.* The experiments were made not later than 2 hr. after collections, and may thus be supposed to give a good approximation to the respiration of the species in the lake at the various seasons. The results shown in Fig. 6 are combined from two successive years.

Fig. 6 shows that the oxygen consumption found may vary from 1·7 (measured in December at 7·0° C.) to 4·8 μl./hr./individual of 20 mg. (beginning of August at 19° C.). The dotted curve shows what the oxygen consumption would have been if it had varied according to Krogh's curve. This computation was based on the oxygen consumption found at the end of October at 10·2° C., and on the differences between this temperature and experimental temperatures. It will be seen that the measured and computed oxygen consumptions are nearly the same from August to December (and most probably during the whole winter). In June, however, the measured oxygen consumption is *c.* 30% higher than that computed according to Krogh's curve. It is thus in the main found to be the same for the animals from the lake Rørbaek Sø as for the animals from the river Funder Aa (Fig. 3).

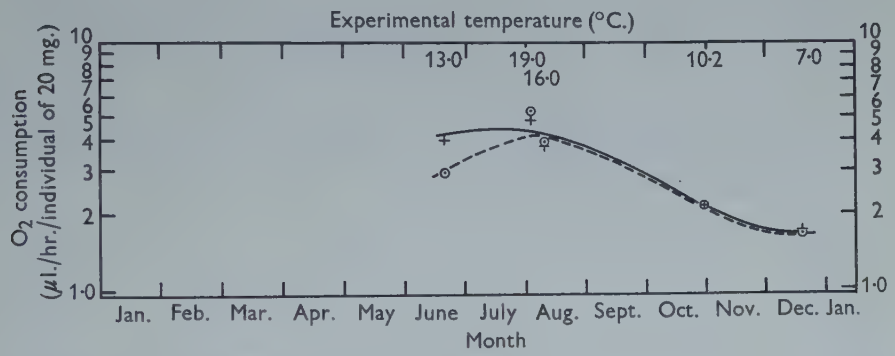

Fig. 6. Oxygen consumption of *A. fluviatilis* from Rørbaek Sø at various seasons (+———+) determined 2 hr. or less after collection of the animals and at the various temperatures of the lake. The experimental temperatures are indicated above. Hypothetical oxygen consumption (⊙ - - - - - - ⊙) based on the determination from October and computed in accordance with Krogh's curve. For further explanation see text.

(*e*) *Experiments carried out with limpets from Rørbaek Sø at the same experimental temperatures,* 18° *and* 11° C., *during all seasons.* The animals were placed in a thermostat at the experimental temperature chosen a short time after collection, and the determinations of the oxygen consumption were made in the period *c.* 2–25 hr. after collection. The results are shown in Fig. 7; when several experiments were carried out during the same period of 2–25 hr. only their mean is indicated.

The curves in Fig. 7 seem to show that the oxygen consumption found at 18° C. is *c.* 1·7 times higher than that found at 11° C., i.e. not quite as high as expected according to Krogh's curve. The oxygen consumption found during summer (at 11° C.) is higher than that found in autumn. Altogether the curves are similar to the curves indicating the oxygen consumption of the limpets from the river Funder Aa (Figs. 4, 5). (Perhaps the respiratory maximum of the lake animals in spring–summer was a little more protracted than that of the river animals.)

(*f*) *Experiments made with limpets from another lake called Borresø and carried out at two fixed temperatures,* 18° *and* 11° C., *during all seasons.* Thus, the experiments were similar to those just mentioned on animals from Rørbaek Sø, except that they were made in the period 17–26 hr. after collecting.

The curves in Fig. 8 are much the same as those in Fig. 7. There occurs a distinct spring maximum of the oxygen consumption, but perhaps this is barely as high as in the case of the river animals (cf. Figs. 8 and 4).

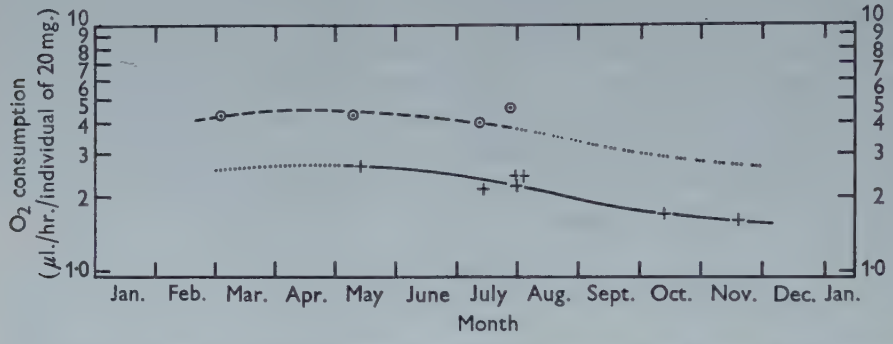

Fig. 7. Seasonal variation of oxygen consumption of *A. fluviatilis* from Rørbaek Sø determined 2–25 hr. after collection of the animals and at two constant experimental temperatures, 18° C. (⊙ - - - - ⊙) and 11° C. (+———+).

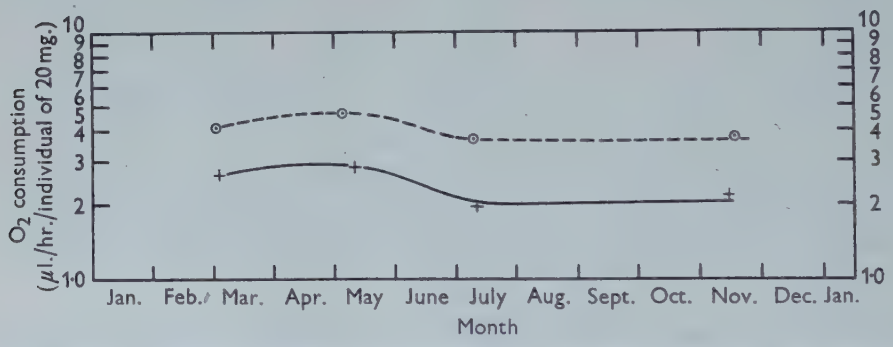

Fig. 8. Seasonal variation of oxygen consumption of *A. fluviatilis* from Borre Sø determined *c.* 17–26 hr. after collection of the animals and at two constant temperatures, 18° C. (⊙ - - - - ⊙) and 11° C. (+———+).

All in all the series of experiments mentioned above clearly show that there occurs a seasonal variation of the rate of respiration of *A. fluviatilis* both in lakes and streams; in spring and early summer the rate of respiration is higher than at other seasons of the year.

IV. SEASONAL VARIATION IN THE OXYGEN CONSUMPTION IN RELATION TO REPRODUCTION AND GROWTH

The rate of respiration showed a distinct maximum during spring and early summer. It is supposed that the maximum rate is caused by reproductive activity, including ripening of eggs and sperm, before the beginning of egg laying. It might be, however, that an increased growth rate in spring and early summer accounted for the maximum rate of respiration at this time. Yet on comparing our results with

those obtained by Hunter (1953) on growth and egg laying period of *A. fluviatilis*, this explanation becomes improbable.

Hunter studied the shell growth of the species in a Scottish stream, Upper Craigton Burn. The physiographical conditions of this stream are, according to Hunter's description, very similar to those prevailing in Funder Aa. This applies to the seasonal change of temperature, e.g. the observed maximum and minimum temperatures, and the current rate. Furthermore, the period of reproduction of the limpets in Upper Craigton Burn coincides with the reproduction period in Funder Aa. A comparison between Hunter's studies on shell growth and our studies on the respiration is therefore considered very reasonable.

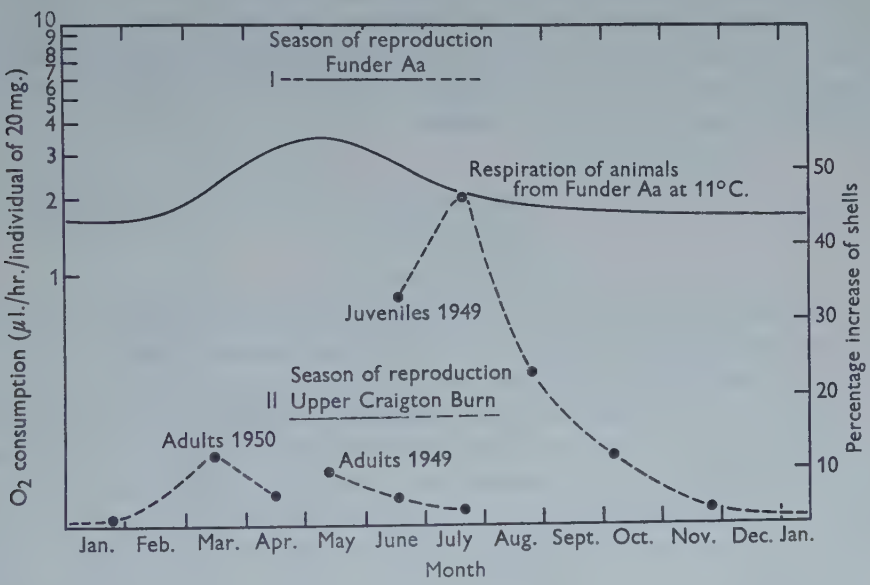

Fig. 9. Seasonal variation of rate of respiration of *A. fluviatilis* from Funder Aa, Jutland, at 11° C. (solid line), and the growth rate of the same species from Upper Craigton Burn, Scotland (broken line) indicated in percentage increase of shell length per month (data from Hunter, 1953). Lines I and II indicate season of egg laying of the two populations. For further explanation see the text.

In Hunter's paper is indicated the mean length of the shells of the limpets at various seasons, both the means of the generation from 1948–9 and from 1949–50. On the basis of these indications we computed the percentage increase of shell length during periods of 30 days, taking the mean shell length at the beginning of every period as a starting point. The results of this computation of the growth rate is shown in Fig. 9 (broken line).

For comparison the respiration of *A. fluviatilis* from Funder Aa at 11° C. is also given in Fig. 9 (solid line), and the reproduction periods of both populations are likewise shown.

Fig. 9 shows clearly that the growth rate of the shells of young animals is highest just after their development from the eggs in summer, then it decreases in autumn

and is very low during winter. In early spring, March, the growth rate of the animals (now adults) is increased, but then it decreases again during spring and summer, just at the time when the animals are sexually mature.

From a comparison between the curves of the growth rate and the rate of respiration it must be concluded that the increased rate of respiration during spring and early summer cannot be accounted for by an increased growth rate since this, on the contrary, decreases at that time. But (*a*) the time of the change of the growth rate (March, adults), and (*b*) the time of the increase in rate of respiration (March–April), compared with (*c*), the beginning of the reproduction period of both populations (April), warrant the conclusion that reproduction is responsible for both the change in growth rate and the rise in rate of respiration.

No respiration experiments on juvenile limpets have been carried out, i.e. with animals of less than 3–4 mg. live weight. The very rapid growth of juveniles found by Hunter makes such experiments attractive, especially for comparison with the respiration of older and mature individuals.

V. OXYGEN CONSUMPTION UNDER VARIOUS EXPERIMENTAL CONDITIONS

(*a*) *Partial starvation*

The usual way to treat poikilothermic animals required for respiration experiments is to place them in a thermostat at the desired temperature; after some time of adaptation the respiration experiments are then carried out with selected individuals.

In the case of *A. fluviatilis*, 50–200 individuals just collected were put into bottles containing 150–200 ml. of aerated water, and the bottles were placed in a thermostat at the desired temperature. The animals were then picked out in suitable numbers when the determinations of the rate of respiration were to be made. When, after collection, the animals were kept together in the bottles in the thermostat, they were often seen eating algae on one another's shells. In spite of that they must be regarded as partly starved.

After this treatment of the limpets it was expected at the outset (1) that a few hours after collection it would be possible to find a fairly constant oxygen consumption at the chosen temperature, and (2) later on, owing to the influence of partial starvation, a reduced consumption. In order to investigate whether both expectations were realized several series of experiments were carried out in the period between 2 and 75 hr. after collection of the animals from the river Funder Aa.

The results of three series of experiments are illustrated in Fig. 10, A carried out in July 1953 at 18° C., B carried out in June 1954 at 18° C., and C also carried out in June 1954, but at 11° C. Furthermore, the results of a series D from October 1954 carried out at 11° C. are added at the bottom, but for these, the treatment differed somewhat from that just described; the *same* experimental animals were used in all experiments of this series (cf. p. 45), and in the time between the experiments the animals were stored in aerated water in their respiration bottles (*c.* two to nine individuals in each bottle of 4–6 ml.) in the thermostat at 11° C. Therefore,

the animals in series D have had little opportunity of eating algae growing on the shells of other animals, less than the animals used for series A, B and C, which were picked out successively from among 50–200 individuals.

As soon as the limpets arrived at the laboratory after collection, and before the experiments of series A–D were started, a determination of the oxygen consumption at the temperature of the river was carried out. These experiments were made as described on p. 44, but each comprised ten single determinations; the results are indicated in Fig. 10 along the ordinate, and the temperatures of the river at the time of collecting are shown also.

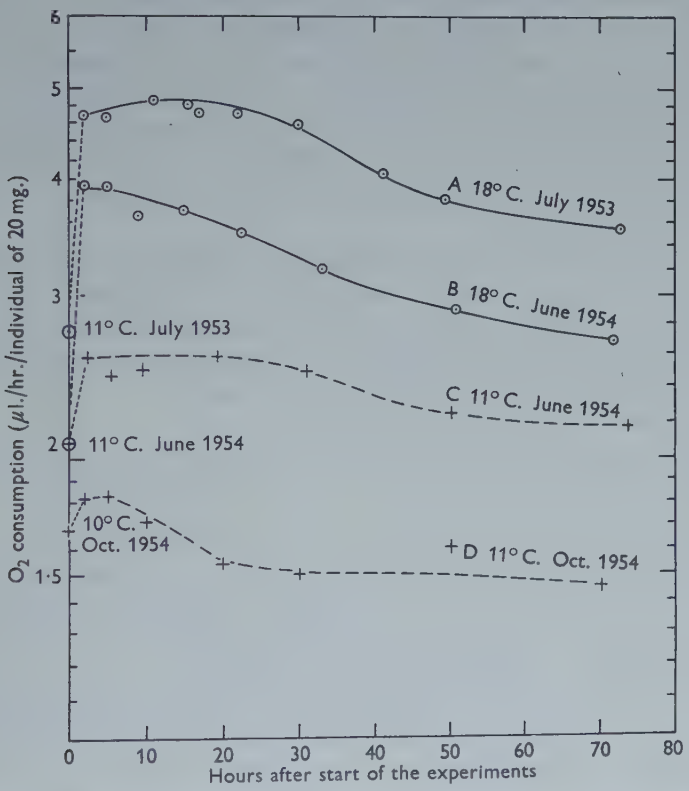

Fig. 10. Oxygen consumption (series A–D) of *A. fluviatilis* from Funder Aa in relation to partial starvation and determined at constant experimental temperatures (11 and 18° C.) differing from the various river temperatures. Oxygen consumptions indicated on the ordinate itself were determined at the same times as series A–D, but at river temperatures and within 2 hr. after collection of the animals; their points are connected by a dotted line with the first point of the series to match.

The results shown in Fig. 10 call for the following remarks.

Series A. In this case there was a fairly constant oxygen consumption of *c.* 4·8 μl. O_2/hr./individual of 20 mg. in experiments carried out 2–20 hr. after collecting, and then a decrease to *c.* 3·5 μl. *c.* 73 hr. after collecting. Thus, 3 days after collection the oxygen consumption had decreased to about two-thirds.

Further series of experiments of the same kind as series A and carried out at various seasons have shown that during partial starvation the variation of the oxygen consumption in the period 2–20 hr. after collection is rather slight, but the consumption is not constant.

Series B, experiments also carried out at 18° C., is an example of a slight decrease of the oxygen consumption in the period 2–20 hr. after the beginning of the experiments. The decrease during the whole period 2–72 hr. after collection is from *c.* 3·9 to 2·7 μl. O_2/hr./individual of 20 mg., i.e. to about two-thirds. This decrease is the same as for series A. But the rate of respiration of series A is higher than that of series B, a difference presumably caused by seasonal variation.

Series C is coincident with series B, but as already mentioned carried out at a temperature of 11° C., nearly the temperature of the river Funder Aa at that time. The oxygen consumption during the period 2–20 hr. after collecting does not seem to vary much. After that it decreased slowly from *c.* 2·6 to 2·2 μl. O_2/hr./individual of 20 mg. at 73 hr. after collection. It is a decrease to about four-fifths. Thus, the decrease at 11° C. in series C was not as great as the decrease in the contemporary series B at 18° C.

Series D. In the period 2–5 hr. after the beginning of these experiments the oxygen consumption was *c.* 1·8 μl./hr./individual of 20 mg.; the decrease commenced very soon and after 70 hr. it was *c.* 1·45 μl., i.e. a decrease to about four-fifths. Therefore in series D, where the animals starved more than the animals of the series A, B and C, the decrease of oxygen consumption set in earlier.

Altogether series A–D have shown:

(1) Partial starvation appears to have a considerable influence on the rate of respiration of the limpets.

All four curves showing the rate of respiration have about the same form; they show a comparatively high, but not constant oxygen consumption during the first hours after collection, then, in consequence of partial starvation, a distinct decrease of the oxygen consumption, and a slighter decrease 50 hr. after collecting.

(2) During partial starvation the decrease of the oxygen consumption is greater at high experimental temperature (series A and B) than at low temperature (series C and D); compare especially the contemporary series B and C, where there is a greater difference between the two curves at the beginning of the experiments than after 3 days.

(3) When the partial starvation is severe the decrease of the oxygen consumption commences earlier than under somewhat better food conditions (series D compared with A, B and C).

(4) The differences between A and B, and between C and D are probably a seasonal effect.

The curves of series A and B seem to show that the oxygen consumption at 18° C., 2–20 hr. after the start of the experiments, is 1·7–1·8 times greater than the oxygen consumption found at the same time at 11° C., the river temperature. This is not quite as much as expected according to Krogh's curve (*c.* 2·0).

It is seen that in series C the oxygen consumption is a little greater 2–20 hr. after the start of the experiments than just after collecting, presumably because of activity.

(*b*) *Feeding from alga-covered stones*

The results of the experiments on partial starvation raised the question: if the experimental animals were able to take food in the usual way from alga-covered stones, would they then perhaps have a constant or nearly constant rate of respiration? In order to test this a series of experiments of the same kind as described under (*a*) were carried out, but in this case the animals were kept up to 70 hr. on alga-covered stones in an aquarium. The stones were taken from the collecting place in the river Funder Aa at the same time as the limpets. As soon as the animals arrived at the laboratory they were placed on the stones in an aerated aquarium and stayed there during the whole period of experiments, except during the hour in which the oxygen consumption was determined. The temperature of the aquarium was raised step by step (cf. p. 62) from 9° C., the river temperature, to 18° C.

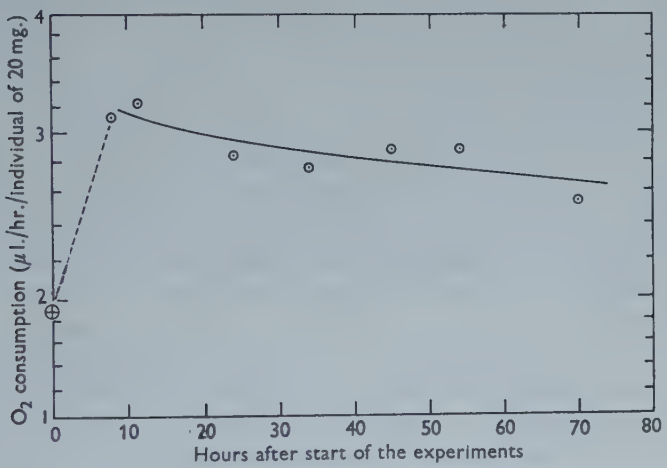

Fig. 11. A slight decrease of oxygen consumption of *A. fluviatilis* kept on alga-covered stones before and during the series of experiments; experimental temperature 18° C. On the ordinate, oxygen consumption at 10° C., about river temperature, and determined within 2 hr. after collection of the animals, October 1955.

The animals were collected in October 1955 and brought to the laboratory, where, for comparison, an experiment at about river temperature (10° C.) was carried out without delay.

The results of the series of experiments at 18° C. are represented in Fig. 11, together with the results of the experiment at 10° C. The curve shows that the rate of respiration is reduced from *c.* 3·2 to *c.* 2·6 µl. O_2/hr./individual of 20 mg. during the time of the experiments, that is to say, to about four-fifths. If the curve is reliable (another determination 70 hr. after the beginning of the experiments would have been desirable) the decrease of oxygen consumption found after 3 days is smaller than in the case of partial starvation, though distinct (see Fig. 10).

The decrease in oxygen consumption is presumably caused by the living conditions not being quite ideal, although the animals in this series of experiments were able to take food. Perhaps the alga-covering on the stones changed during the experiments; furthermore, the water-movements in the aquarium are very different from the current in the river. Thus in this case also it may be supposed that the rate of respiration is influenced by a scarcity of food.

It will be noted from Fig. 11 that the oxygen consumption at 18° C. at the beginning of the experiments, *c.* 3·2 μl. O_2/hr./individual of 20 mg., is about 1·6 times as great as the consumption at 10° C. just after collection.

(*c*) *Complete starvation*

On account of the strong and fairly rapid influence of partial starvation on oxygen consumption mentioned above, it was decided to investigate the influence of complete starvation. In order to avoid any influence of a temperature change the experiments were carried out at about the same temperature as that found in the river Funder Aa at the time of the experiments.

The experiments were made in July 1955 at 13° C. The temperature of the river varied during the experimental period from 12·2 to 14·6° C., with an average of *c.* 13·5° C. To make sure that the animals took no food during the whole series of experiments the following method was adapted: just after collection each individual *A. fluviatilis* was placed in a little glass tube closed at both ends by means of coarse nylon-gauze. The glass tubes were joined in groups, each group comprising individuals enough for one experiment; the groups of glass tubes were numbered, put in aluminium boxes with no end-walls, but closed by means of coarse nylon-gauze. The boxes containing the glass tubes were then placed in the river so that the longitudinal axes of the tubes and cases coincided with the direction of the current. In this way the experimental animals were kept *in their natural environment*, and starved completely during the whole experimental period. At intervals, when required for an experiment, a group of animals was picked out from a box in the river and brought to the laboratory where the determination of the oxygen consumption was made within 2 hr. of collection.

The series of experiments were made after a starvation period of 0 to 96 hr. (Fig. 12*A*). For purposes of comparison, two other determinations were made:

(1) Oxygen consumption of animals kept in glass tubes in the river as described above and totally starved for 96 hr., and then placed on alga-covered stones in a box in the river for 3 days (Fig. 12*B*).

(2) Oxygen consumption of animals partly starved when kept together as usual in great numbers in an aquarium (p. 56) in the laboratory during the same period as the experimental animals of the main series starved completely in the river (Fig. 12*C*).

Curve *A* in Fig. 12 shows that the oxygen consumption at the beginning was 3·4 μl./hr./individual of 20 mg. and then during starvation *decreased rapidly* in the period 0–20 hr. after the start of the series. After this period the decrease continued till the end of the period, but apparently in diminishing degree. 96 hr. after the

beginning of the starvation the oxygen consumption had declined to 1·95 μl./hr./individual of 20 mg., i.e. to about three-fifths of the initial value.

In the control experiments the oxygen consumption of animals first starved for 96 hr. and then placed on alga-covered stones (point *B*) has increased considerably, namely, to 2·7 μl. O_2/hr./individual of 20 mg. Nevertheless, it is not the same as that in the beginning of series A.

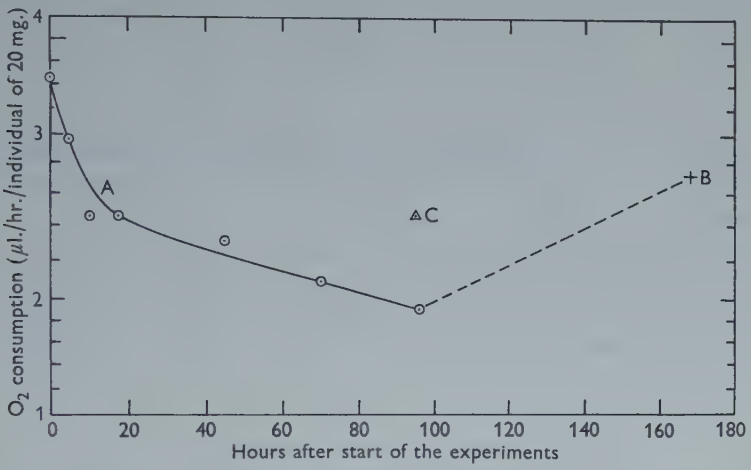

Fig. 12. ⊙———⊙ *A*; oxygen consumption of *A. fluviatilis* from Funder Aa during complete starvation, kept singly in the river, at 13° C. (about river temperature). + *B*; oxygen consumption of individuals starved singly in the river for 96 hr. and then kept for 72 hr. on alga-covered stones in the river. △ *C*, oxygen consumption of *A. fluviatilis* partly starved in an aquarium, July 1955.

The oxygen consumption of the animals partly starved for 95 hr. at 13° C. is 2·5 μl. O_2/hr./individual of 20 mg. (point *C*). It thus lies between the rate of respiration of animals starved completely and that of animals starved completely and then fed on alga-covered stones.

A comparison between the oxygen consumption of animals affected by complete starvation (*A*, Fig. 12) and animals affected by partial starvation (Fig. 10), or by some food intake from alga-covered stones (Fig. 11), shows how quickly the influence of complete starvation began to manifest itself. There was no time at the beginning in which the completely starved animals had a nearly constant oxygen consumption as in the other cases. The importance of food intake for the rate of respiration of the limpets is thus clearly demonstrated.

The foregoing series of experiments, in which the animals were kept singly in glass tubes in their natural environment in Funder Aa and completely starved during periods of $5\frac{1}{2}$–96 hr., was carried out from the 28th to the 31st of July when the river temperature was about 13·5° C. The number of egg capsules produced under these circumstances was observed. The result is summarized in Table 3.

The number of animals in each experiment is about the same. As the time of starvation increased from $5\frac{1}{2}$ to $26\frac{1}{2}$ hr. the number of egg capsules produced in

Table 3

No. of animals in each experiment	Time of starvation (hr.)	No. of egg capsules produced
22	5½	1
23	10	2
24	17½	9
22	26½	16
23	45	4
24	70	17
24	96	16

each experiment increased. But after that the number did not increase further; it must, therefore, be presumed that after starvation for about 24 hr. the production of egg capsules nearly stopped.

Furthermore, it may be computed, on the basis of the starvation for 17½–26½ hr., that one limpet (live weight *c.* 30 mg.) produces one egg capsule during *c.* 36–48 hr. It would seem that egg laying takes place most frequently during night (cf. Bondesen, 1950), and more egg capsules seem to be produced by larger than by smaller individuals.

(*d*) *With gradual or abrupt temperature increase*

It is of general importance to know how the oxygen consumption of poikilothermic species varies according to changes of temperature. This is the case when a comparison is required, e.g. between the oxygen consumption of various species, or of various populations or races of the same species, or of the same species at various seasons. The experiments must then often be carried out at a common temperature differing from the temperature of all or some of the habitats, and it is therefore necessary to expose some of the experimental animals to fairly large alterations of temperature.

In the case of *A. fluviatilis*, oxygen consumption in relation to temperature (Berg, 1952, 1953) sometimes followed the so-called Krogh's curve (1914, 1916) indicating the relationship of oxygen consumption to temperature for various species when at rest. But in other cases the variation was not quite so great (cf. pp. 52, 53, 58, 60, 66). The question therefore arose of how the oxygen consumption is affected by a number of small temperature increments compared with a sudden change; and of how the rate of respiration is influenced by a change of temperature far greater than the daily temperature variations of the habitat. When does the oxygen consumption vary in accordance with Krogh's curve?

(A) In order to answer these questions three series of experiments with the limpets from Funder Aa were carried out. Throughout each series, the same experimental animals were used (cf. p. 45).

The first series was performed in October 1954. The temperature of the river was 7·8° C. Immediately after the animals were brought to the laboratory an experiment was started at 9° C. The temperature was then increased *c.* 1° C. nearly

every hour, and the oxygen consumption measured 1, 2, 3, 4, 5, $6\frac{1}{2}$, $7\frac{1}{2}$, $8\frac{1}{2}$ hr. after starting the experiments. The results are shown by means of triangles in Fig. 13.

The second series was also carried out in October 1954. In this case the increase of the experimental temperature was abrupt from the river temperature of 8·5 to 18° C. The determinations of the oxygen consumption were carried out at the last mentioned temperature nearly every hour after starting the experiments and the results are shown by means of circles in Fig. 13; their mean is indicated by a horizontal line.

The third series was carried out in December 1954 during a number of temperature increments, 1° C. per hour, from the river temperature of 5·7 to 15° C. Determinations of oxygen consumption were made every hour as in the first series. The results are shown as crosses in Fig. 13. In this figure a Krogh's curve is drawn for comparison.

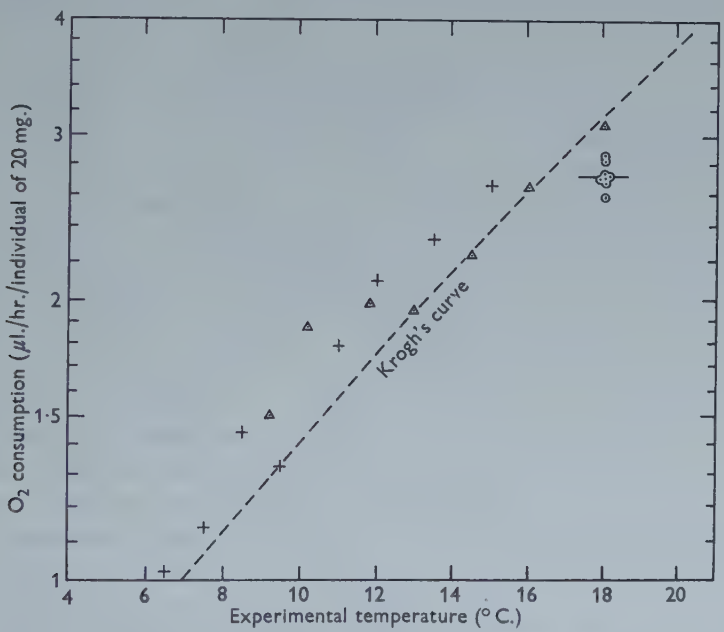

Fig. 13. Oxygen consumption of *A. fluviatilis* in relation to gradual versus abrupt increase of the experimental temperature from the temperature of the river. △, first series, after stepwise increase of temperature to 18° C., October 1954. ⊙, second series, after abrupt increase of temperature to 18° C., October 1954; the horizontal line indicates the mean of the consumptions of the second series. +, third series, after stepwise increase of temperature, December 1954. - - - - -, Krogh's curve. With gradual increase of temperature the oxygen consumption follows mainly Krogh's curve, by an abrupt increase it is not quite as high.

The three series of experiments give the following results:

(*a*) With a number of small temperature increments the oxygen consumption of the limpets increases immediately after the change of temperature, and the increase of oxygen consumption follows in the main a Krogh's curve. In the series from October the values at the beginning are a little too high; but the later values follow

a Krogh's curve and this is also the case with the values from December. (*b*) With an abrupt increase of temperature (second series, October) the oxygen consumption at 18° C. is not quite so high as in the case of a gradual increase to this temperature (first series, October).

At 18° C. the difference between the value of oxygen uptake of the gradual series and the mean value of the abrupt series is *c.* 0·43 μl. O_2. There is a 95 % probability that the first-mentioned value deviates at most ±4 % or 0·12 μl. from its true value (cf. p. 48), and the uncertainty of the mean value is, of course, still less. Thus there is a reliable difference between the two series.

After an abrupt rise of temperature to 18° C. the oxygen consumption did not reach the level attained after a gradual increase in temperature, even though measurements were continued for 8–9 hr. after the abrupt rise. This is presumably a result of some interest for experimentalists. Only when the change of temperature is brought about slowly (1° C. per hour), will the rate of respiration change according to Krogh's curve.

The influence of the rate of change of temperature on the oxygen consumption was unknown at the beginning of our experiments. Some of the results concerning seasonal variation (p. 51) may be influenced a little by a too rapid change in the experimental temperature.

In the series of experiments described the whole change of temperature was about 10° C. This is far more than the daily temperature change of Funder Aa, which is seldom ±2° C. and often less. The experimental change of temperature, however, is not much greater than the seasonal change of temperature of Funder Aa (Fig. 2), but the experimental change covers higher levels, though not higher than the species can tolerate in Rørbaek Sø.

(B) Further experiments on the influence of gradual temperature increases on the oxygen consumption showed, however, that in these cases the increase of oxygen consumption is not always as great as expected according to Krogh's curve.

A series of experiments was made in December 1954 with the limpets from Rørbaek Sø. In this case, too, the same experimental animals were used throughout the gradual increase of the temperatures, 1° C. per hour. The temperature of the lake was 5·9° C. The experiments started at 7° C. immediately after the animals were brought to the laboratory, and measurements of the oxygen consumption were then carried out each hour till the temperature had increased to 15° C.

The result of the experiments is shown in Fig. 14. The oxygen consumption of an animal of 20 mg. is indicated as usual (*I*, continuous, heavy curve), but in this case each of the five separate determinations of each experiment (cf. Fig. 1, p. 46) is also shown (nos. 1–5), in order to show how regular the increase of the oxygen consumption of all the specimens was.

From Fig. 14 it is seen that the oxygen consumption of an individual of 20 mg. increases with increasing temperature, but not quite so much as expected according to Krogh's curve. With an increase of temperature from 7 to 15° C. the oxygen consumption of the 20 mg. individual is increased 1·9 times, but according to Krogh's curve it ought to have been increased *c.* 2·4 times. Thus a gradual increase

of temperature does not guarantee that the oxygen consumption increases according to Krogh's curve.

(*e*) *In still or shaken bottles*

In earlier experiments lasting 1 hr. it was regarded as advantageous to shake the respiration bottles every 10 min. in order to move the water surrounding the animals. Later on this was omitted, because the animals were presumed to move so much that no water layer depleted of oxygen could be formed around them so as to

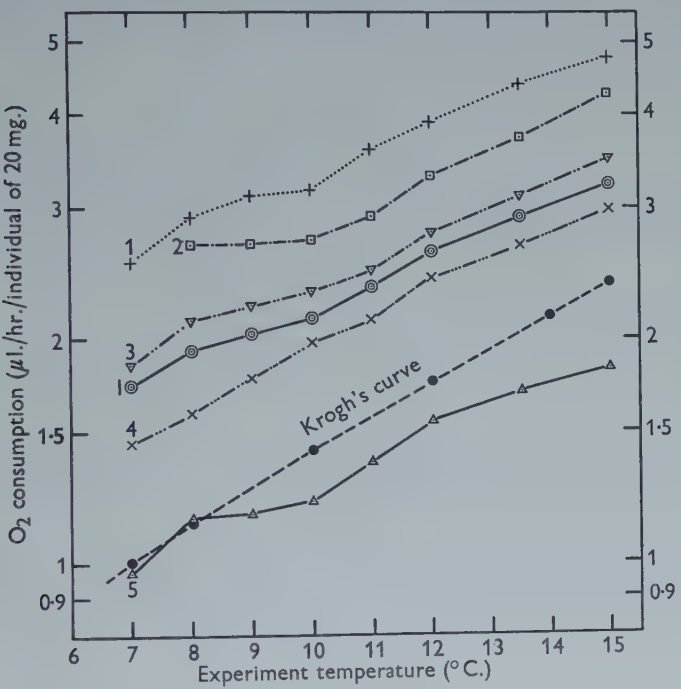

Fig. 14. Oxygen consumption of *A. fluviatilis* in relation to stepwise increase of the experimental temperature from the lake temperature. I———, oxygen consumption of an individual of 20 mg., December 1952. 1–5, oxygen consumption according to the five separate determinations constituting an experiment (cf. Fig. 1, p. 46); the live weights were 33·6, 31·9, 23·6, 16·9 and 8·5 mg., respectively. - - - - -, Krogh's curve. The oxygen consumption increases with increasing temperatures, but not quite as much as expected according to Krogh's curve.

diminish their oxygen consumption. But the problem remained unsolved. It was, therefore, decided to test whether or not movement of the water surrounding the experimental animals would increase the oxygen consumption. It was planned, furthermore, that the experiment should be carried out at two different temperatures in order to ascertain if stirring of the water would influence the relation of oxygen consumption to temperature.

For comparison four experiments were carried out in August 1956, when the temperature of Funder Aa was 10·6° C. They were made immediately after collection of the animals, at a temperature of 11° C., and followed one another without

delay. In the two experiments the bottles containing the limpets were shaken every 6–7 min., while the two other bottles were kept still in the thermostat as long as the experiment lasted, i.e. for 1 hr.

After determinations of the oxygen consumption at 11° C. the temperature of the thermostat was gradually increased in the course of 5 hr. to 18° C., and the animals were kept at the last-mentioned temperature for $1\frac{1}{2}$ hr. before the determinations of their oxygen consumption during 1 hr. were made. This time the bottles of the first two experiments were kept still, and the bottles of the other two experiments were shaken every 6–7 min. The result of the experiment in Table 4 shows the oxygen consumption (μl./hr./individual of 20 mg).

Table 4

	Respiratory bottles shaken	Respiratory bottles kept still
Experimental temperature 11° C.	1·91 2·04	1·75 2·05
	Mean 1·98	Mean 1·90
Experimental temperature 18° C.	3·35 3·21	2·90 3·07
	Mean 3·28	Mean 2·99

Thus shaking seems to have an effect; in stirred water the oxygen consumption seems to be greater than in still water. At 18° C. the effect of shaking may be about 10%.

The increase of oxygen consumption in relation to the temperature increase from 11 to 18° C. is about the same for shaken and not shaken bottles, *c.* 1·6. According to Krogh's curve (1914) it ought to be *c.* 2·0 (cf. Berg, 1953).

VI. OXYGEN CONSUMPTION IN RELATION TO BODY WEIGHT

As previously mentioned (p. 45), it was supposed—on the basis of a series of preliminary experiments—that the rate of respiration in relation to the weight of the individuals was of such a kind that the slope of the regression line in a logarithmic co-ordinate system was 0·73. It would be reasonable by means of part of the numerous experiments which have gradually been carried out, to verify this assumption. The statistician, civil engineer H. T. Stenby, has kindly made the following calculations.

(*a*) If a respiration experiment of the type described is made in such a way that disturbing influences only make themselves felt in a slight degree, the measured values of oxygen consumption will be placed nearly on a straight line, and the correlation coefficient will tend towards 1. When this is the case it can be shown that the slope of the regression line for the oxygen consumption in relation to the weight of individual approaches a fixed value. For nine series of experiments the average slope (i.e. tangent of the angle of inclination α) is indicated in Fig. 15 for each series

as a function of the average correlation coefficient of the series. The calculations have been made in two ways, partly by assumption of the y values as the only variable, partly by assumption of both y and x values as variables (orthogonal regression, cf. Hald, 1948). The results are shown in Fig. 15 by circles and crosses, respectively. It will be seen that, using both ways of calculation, the inclination, tan α, tends towards 0·70–0·75 when the correlation coefficient grows towards 1; it means that the more the experiments are undisturbed by irrelevant factors the more the inclination itself approaches the value mentioned.

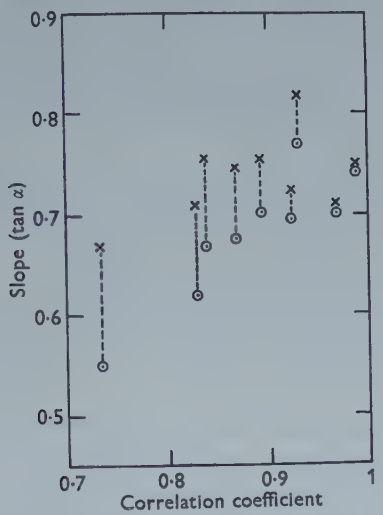

Fig. 15. The slopes (tan α) of regression lines (showing oxygen consumption in relation to weight) in proportion to correlation coefficients. ⊙, ratio when oxygen consumption, y value, is regarded as the only variable. ×, ratio when both oxygen consumption, y value, and weight, x value, are regarded as variables (orthogonal variation). When the correlation coefficients increase to nearly 1, the slopes tend towards 0·70–0·75.

(*b*) Nine series including altogether sixty-seven experiments of the type shown in Fig. 1 (a total of 333 determinations of oxygen consumption) have been subjected to a statistical calculation. They all had a correlation coefficient for log x, log y, which was $>0{\cdot}8$. The calculation showed that the middle inclination for the sixty-seven experiments was tan $\alpha = 0{\cdot}71$. Thus the estimated value of 0·73 only deviates inconsiderably from the average found here.

(*c*) On the basis of three series of experiments with a total of twenty-two experiments (i.e. 110 determinations of oxygen consumption) the common inclination of the regression lines $y = a + bx$ for these three series of experiments has been calculated (cf. Hald, 1948) to be 0·716. Thus, this result also deviates very little from the inclination used, 0·73. The standard deviation for the inclination calculated is $\pm 0{\cdot}034$. In this way there is 68% probability for the conclusion that the true inclination is $0{\cdot}716 \pm 0{\cdot}034$, i.e. it is between 0·68 and 0·75; thus this statement of the variation of the inclination of the regression line includes 0·73, the estimated value used.

VII. DISCUSSION

Seasonal variation of respiration. The variation of the oxygen consumption of *A. fluviatilis* in the course of the year is great. Measured at the same temperature the oxygen consumption is about 1·3 to nearly twice as much in spring and summer as in autumn and winter (cf. Figs. 4, 5, 7 and 8). This increase of oxygen consumption during the reproductive period is regarded as an expression of the sexual activity.

Other freshwater molluscs, and perhaps also other freshwater invertebrates, have presumably a similar increased oxygen uptake during their reproduction periods. For that reason, and also because the oxygen consumption varies during the reproductive period (as shown by *A. fluviatilis*) a closer examination of the difference between the rates of respiration of various species is difficult. Environmental influences on the oxygen consumption may often be smaller than the influence of reproduction on the respiration. This conclusion is probably of some importance from an ecological point of view. When comparing the oxygen consumption of different species living in different habitats or of the same species from different localities, the possible influence of the reproduction must be considered. Perhaps this influence conceals differences in oxygen consumption which, outside the reproduction periods, would be easily recognizable and in accordance with the variations of the environment.

The seasonal temperature variations of the habitats of the limpets are fairly considerable, especially in Rørbaek Sø (Fig. 2, p. 49). For that reason one might have expected a temperature adaptation of the rate of respiration (cf. Krogh, 1916; Wells, 1935*a*, *b*; Spärck, 1936; Thorson, 1936; Edwards & Irving, 1943*a*, *b*; Berg, 1953; Scholander, Flagg, Walters & Irving, 1953; Bullock, 1955) so that the rate was comparatively low in summer (or comparatively high in winter) compared with the variations according to Krogh's curve. The observed seasonal variation of the oxygen consumption of the limpets does not show the existence of such an adaptation. In summer, on the contrary, the oxygen consumption is high.

Earlier experiments with *A. fluviatilis* from Rørbaek Sø and Funder Aa (Berg, 1953) were carried out in July when the lake had a temperature of *c.* 18° C. and the river *c.* 11° C. The comparative experiments were carried out at 11 and 18° C. for both the form from the warm water of the lake and the cold water of the river. The experiments showed that the animals from the summer warm environment (18° C.) had a higher oxygen consumption than those from the summer cold environment (11° C.).

Since the earlier experiments were carried out our way of using the polarometric method has been improved, but the earlier measurements must still be regarded as quite reliable. But how does our knowledge of seasonal variation affect our estimate of these early results?

Because the seasonal variation of the oxygen consumption is so great that the oxygen uptake in summer may be twice as great as in winter (at the same temperature), and because the oxygen consumption varies greatly in summer, this season must

now be regarded as unsuited for comparison between the rates of respiration of the two populations. It may then be asked whether winter is more suitable. Unfortunately, we possess too few experiments on the oxygen consumption of the limpet population from Rørbaek Sø to answer the question completely, but the following may be noted:

(*a*) In Fig. 16 the oxygen consumption of the population from Funder Aa is shown according to measurements from October to February at the same temperature as found in nature and immediately after the limpets were brought to the laboratory. It will be seen from the figure that the oxygen consumption of this form in winter at 7° C. is *c.* 1·1 ml./hr./individual of 20 mg. An experiment in December with the Rørbaek Sø population at the same temperature and also carried out immediately after collecting the animals gave the result 1·7 ml./hr./individual of 20 mg.

(*b*) In seventeen experiments with the population from Funder Aa carried out from October to February at 11° C. and ½–10 hr. after collecting, the limpets showed the mean oxygen consumption of 1·7 ml./hr./individual of 20 mg. (variation from *c.* 1·4 to 2·0 ml.). One experiment with the population from Rørbaek Sø carried out at the same temperature in December and 5 hr. after collecting the animals, gave the value 2·35 ml./hr./individual of 20 mg.

We do not regard these two observations as quite conclusive, but they indicate that outside the reproductive season the form from the lake has a *greater* oxygen consumption than the form from the river.

Owing to the facts that *A. fluviatilis* has no well-defined standard metabolism, and that a complete or partial starvation has a great influence on its oxygen consumption, it may be suggested that in winter the lake form is comparatively well fed. In this connexion it should be added that the growth of the lake form seems to be more rapid than the growth of the river form. At the end of the reproductive season in summer, when the animals die, the live weight of the animals from Rørbaek Sø is often *c.* 80 mg., but from Funder Aa only 40–50 mg.

Oxygen consumption of A. fluviatilis *in relation to temperature*

As is well known, Krogh (1914, 1916) established a curve giving the relation between temperature and the standard metabolism in animals. By the standard metabolism he means the metabolism when conditions involve a minimum activity of the animal and no food is being digested. Krogh's curve shows that with increasing temperature the oxygen consumption increases, but not proportionately. This is seen clearly if the curve is plotted on a logarithmic scale (cf. Berg, 1952).

From earlier experiments (Berg, 1952) it was concluded that below 18° C. the increase in oxygen consumption of the limpets with increasing temperature is the same as according to Krogh's curve, but at higher temperatures than *c.* 18° C. the increase is smaller. According to Krogh's curve an increase of temperature from 11 to 18° C. will cause the oxygen consumption to increase *c.* 2·0 times. Experiments with the limpets both from Funder Aa and Rørbaek Sø (Berg, 1953) gave the results

c. 1·9 and thus agreed fairly well with the number found according to Krogh's curve.

Some of the experiments published in this paper have augmented the observations on the increase of oxygen consumption caused by a temperature rise from 11 to 18° C. The increase of consumption was found to be 1·6× (p. 52), 1·7× (p. 53), 1·7×–1·8× (p. 58, computed from series A and B, Fig. 10). Thus in these cases the increase of oxygen consumption was not as great as expected according to Krogh's curve. But in some other experiments (p. 62), after a gradual temperature increase, 1° C. per hour, the oxygen consumption in the main followed Krogh's curve. This, however, is not always the case. Exceptions, such as an increase of oxygen consumption only 1·6× after a gradual temperature rise from 11 to 18° C., or from 10° to 18° C., are mentioned above (pp. 66 and 60).

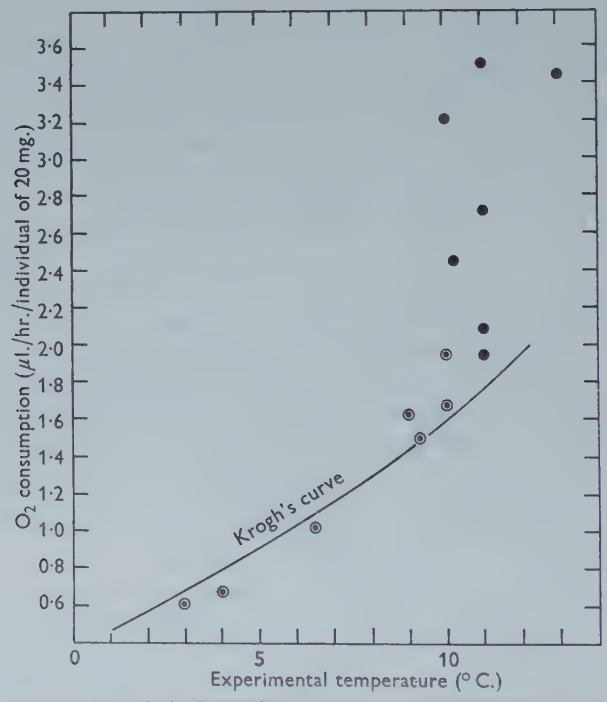

Fig. 16. Oxygen consumption of *A. fluviatilis* in relation to temperature. The experimental temperatures are the same as found in nature, the river Funder Aa, at the various times of the experiments; experiments carried out immediately after the limpets were brought to the laboratory. ⊙, experiments from October to February. ●, experiments from March to September. ———, Krogh's curve.

All in all the oxygen consumption of *A. fluviatilis* in relation to temperature changes cannot be predicted completely. The consumption (below 18° C.) follows Krogh's curve in some cases, especially after a gradual temperature rise, but not after an abrupt rise. It is not, however, always true that a gradual rise in temperature brings about a rise in oxygen consumption as great as that predicted by Krogh's curve.

In two series of the present experiments on seasonal variation (Figs. 3, 6, pp. 50, 53) the oxygen consumption found at various seasons is compared with an hypothetical consumption based on autumn experiments, and computed by means of Krogh's curve and on the various temperatures found during the year. Because the oxygen consumption of the limpets does not always follow Krogh's curve completely at temperatures below 18° C. the hypothetical curves in Figs. 3 and 6 should perhaps be placed a little lower in spring and summer. But this means that the observed oxygen consumption during early spring and summer is even greater than expected, i.e. the seasonal variation of the rate of respiration is still more distinctly demonstrated.

In several experiments with the limpets from Funder Aa the oxygen consumption was determined immediately after the animals were brought to the laboratory and at the different seasonal temperatures of the river. Thus, in these cases the animals were not influenced by a temperature change before the experiments, but must be regarded as adapted to the temperatures in nature. Results of such experiments are to be found in Fig. 16 together with a Krogh's curve. The figure shows that the oxygen consumption in relation to the temperature in October to February, i.e. *outside* the reproduction period, varies nearly according to Krogh's curve. But determinations from March to September during, or just before, the period of reproduction, exhibit higher values than the Krogh curve indicating winter respiration. The figure shows that at *c.* 10° C. and more, the influence of reproduction on the oxygen consumption makes itself very much felt.

VIII. SUMMARY

1. A seasonal variation of the oxygen consumption of the limpet *Ancylus fluviatilis* occurs in populations both from a lake and from a stream. In spring and early summer the oxygen consumption is higher than at other seasons of the year.

2. The increased oxygen consumption in spring and early summer is most probably caused by reproductive activity.

3. A decrease of the growth rate in spring and early summer, computed on the basis of Hunter's observations (1953), must also be regarded as caused by reproductive activity.

4. Partial starvation appears to have considerable influence on the rate of respiration. A comparatively high, but not constant oxygen consumption occurs during the first hours after collection, then, in consequence of partial starvation, a distinct decrease of the oxygen consumption follows, and finally, 50 hr. after collection, a slighter decrease.

5. During partial starvation the decrease of the oxygen consumption is greater at high than at low temperatures. When the partial starvation is severe the decrease of the oxygen consumption commences earlier than under somewhat better food conditions.

6. The oxygen consumption of limpets which had the opportunity of taking food from alga-covered stones in an aquarium decreased after 3 days, but the decrease seems to be less than in the case of partial starvation.

7. The oxygen consumption of the limpets which starved completely decreased rapidly during the period 0–20 hr. after the start of the series of experiments. There was no time at the beginning of the starvation period in which the animals had a nearly constant oxygen consumption. After the period 0–20 hr. the decrease continued, but apparently in diminishing degree; 96 hr. after the beginning of the starvation the oxygen consumption had declined to about three-fifths of the initial value. The oxygen consumption of animals first starved for 96 hr. and then given food when placed on alga-covered stones in the river for 3 days again increased considerably, but not to the initial value.

8. The oxygen consumption in relation to the temperature was found in the main to follow the so-called Krogh's curve from 11 to 18° C. after a gradual temperature increase (*c.* 1° C. per hour), but not after an abrupt increase. The first result confirms earlier observations (Berg, 1952). It does not always seem certain, however, that the increase of oxygen consumption in experiment will follow Krogh's curve completely, even if the temperature increases slowly.

9. Experiments with limpets which may be regarded as adapted to different temperatures in nature show that their oxygen consumption in relation to temperature in October to February, i.e. outside the reproduction period, varies according to Krogh's curve.

10. In shaken bottles the oxygen consumption appears to be greater than in still bottles.

11. The slope of the regression line, tan α, showing the oxygen consumption in relation to weight in a logarithmic co-ordinate system, tends towards 0·70–0·75 when the correlation coefficient, which expresses the dependence of the respiration on weight, increases towards 1 (Fig. 15, p. 67). On the basis of twenty-two experiments the slope of the regression lines has been calculated to be $0{\cdot}716 \pm 0{\cdot}034$.

We wish to express our sincere thanks to the Carlsberg Foundation for grants in support of this study. Thanks are also due to Mr H. T. Stenby, C.E., who made the statistical calculations, and to Dr K. H. Mann, who read and corrected the manuscript.

REFERENCES

BARTELS, H. (1949). Die Bestimmung des physikalisch gelösten Sauerstoffs in biologischen Flüssigkeiten mit der Quecksilbertropfelektrode. *Naturwissenschaften*, **36**, 375.

BARTELS, H. (1950). Die Bestimmung des physikalisch gelösten Sauerstoffs in biologischen Flüssigkeiten mit der Quecksilbertropfelektrode. *Pflüg. Arch. Berlin*, **252**, 264–77.

BERG, K. (1952). On the oxygen consumption of Ancylidae (Gastropoda) from an ecological point of view. *Hydrobiologia*, **4**, 225–67.

BERG, K. (1953). The problem of respiratory acclimatization. *Hydrobiologia*, **5**, 331–50.

BULLOCK, Th. H. (1955). Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev.* **30**, 311–42.

DANSKE HEDESELSKAB (1940). Beretning om hydrometriske undersøgelser.

EDWARDS, G. A. & IRVING, L. (1943*a*). The influence of temperature and season upon the oxygen consumption of the sand crab, *Emerita talpoida* Say. *J. cell. comp. Physiol.* **21**, 169–82.

EDWARDS, G. A. & IRVING, L. (1943*b*). The influence of season and temperature upon the oxygen consumption of the beach flea *Talorchestia megalophtalma*. *J. cell. comp. Physiol.* **21**, 183–89.

HALD, A. (1948). *Statistiske metoder*. København.

HEMMINGSEN, A. M. (1950). The relation of standard (basal) energy metabolism to total fresh weight of living organisms. *Rep. Steno Hosp., Copenh.*, **4**, 7–55.

HUNTER, W. R. (1953). On the growth of the fresh-water limpet, *Ancylus fluviatilis* Müller. *Proc. zool. Soc. Lond.* **123**, 623–36.

KROGH, A. (1914). The quantitative relation between temperature and standard metabolism in animals. *Int. Z. phys.-chem. Biol.* **1**, 491–508.

KROGH, A. (1916). *Respiratory Exchange of Animals and Man*, pp. 1–73. London.

KROGH, A. (1941). *The Comparative Physiology of Respiratory Mechanisms*, pp. 1–172. Philadelphia.

SCHOLANDER, P. F., FLAGG, W., WALTERS, V. & IRVING, L. (1953). Climatic adaptation in arctic and tropical poikilotherms. *Physiol. Zoöl.* **26**, 1, 67–92.

SPÄRCK, R. (1936). On the relation between metabolism and temperature in some marine lamellibranchs and its zoogeographical significance. *K. danske vidensk. Selsk.* **13**, 1–27.

THORSON, GUNNAR (1936). The larval development, growth and metabolism of arctic marine bottom invertebrates. *Medd. Grønland.* **100**, 1–155.

WELLS, NELSON A. (1935*a*). Variation in the respiratory metabolism of the Pacific Killifish *Fundulus parvipennis* due to size, season, and continued constant temperature. *Physiol. Zoöl.* **8**, 318–36.

WELLS, NELSON A. (1935*b*). Change in rate of respiratory metabolism in a teleost fish induced by acclimatization to high and low temperature. *Biol. Bull. Woods Hole*, **69**, 361–7.

ZEUTHEN, ERIK (1947). Body size and metabolic rate in the animal kingdom. *C.R.Lab. Carlsberg* (Sér. chim.), **26**, 17–162.

STUDIES ON THE FEEDING AND NUTRITION OF *TUBEROLACHNUS SALIGNUS* (GMELIN) (HOMOPTERA, APHIDIDAE)

II. THE NITROGEN AND SUGAR COMPOSITION OF INGESTED PHLOEM SAP AND EXCRETED HONEYDEW

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Several workers have tried to ascertain the nitrogenous and carbohydrate materials which are ingested by aphids and coccids by analysing juices, phloem exudates and extracts obtained from the insects' host plants (Evans, 1938; Michel, 1942; Lindemann, 1948; Auclair & Maltais, 1950, 1952; Gray, 1952; Hackman & Trikojus, 1952; Gray & Fraenkel, 1954; Bacon & Dickinson, 1957). It remains to be established, however, whether the insects ingest materials in the same form and at the same concentration as they occur in their host plants. The present paper gives details of a method, outlined by Kennedy & Mittler (1953), for obtaining the sap which normally enters the alimentary canal of feeding *Tuberolachnus salignus* (Gmelin). By determining the nitrogen and sugar composition of this sap and that of the honeydew excreted by the aphid, it was possible to examine the chemical relationship existing between the insect's food and its excreta.

MATERIAL AND METHODS

T. salignus was reared as previously described by Mittler (1957). Standard, 5–6 ft. tall, 2- to 4-year-old, potted *Salix acutifolia* Willd. trees were used as the experimental host plant, except where otherwise stated. Experiments were carried out in a greenhouse at a mean temperature of 20° C. under a photoperiod of 16 hr.

The stem cage. Two split annuli of cork, having an outer diameter of approximately 1·5 in., were attached 3–4 in. from each other on a willow stem. Low-melting-point wax was moulded into gaps between the cork and the willow bark, and into the radial split in each annulus. A sheet of cellophane paper was then stretched in a cylinder about the two annuli, and its slightly overlapping edges clipped together with two 2·5 in. bull-dog paper clips.

Stylet cutting technique and collection of stylet-sap. Twenty adult apterous *T. salignus* were caged on an upright willow stem. 2–3 hr. after the aphids had inserted their stylets into the stem and had excreted several droplets of honeydew, the plant was placed on its side on a bench. The soil in the pot was prevented from

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spilling and from drying out by wrapping the pot in damp sacking. The stem was gently clamped when the plant had been rotated into such a position that some of the feeding aphids were visible in profile when viewed under a binocular microscope.

As *T. salignus* normally presses its head close to the willow stem on which it is feeding, its proboscis was found to extend for at least 0·5 mm. along the surface of the willow's bark from the aphid's head to the point of entry of its stylets into a 2- — 4-year-old stem (Fig. 1 A). A fine splinter of a razor blade, secured to a slender glass rod which was held between the fingers, was brought into position over the proboscis, as indicated in Fig. 1 A, and then pressed sharply against the bark, which acted as a 'chopping-block' for the cutting operation. Resting the hand on the microscope stage or the stem steadied the blade and reduced the risk of dragging the embedded stylets from the stem before the proboscis was severed. Directly after making the cut a clear fluid, termed stylet-sap, exuded from the cut end of the proboscis stump which projected from the stem (Fig. 1 B). On carefully

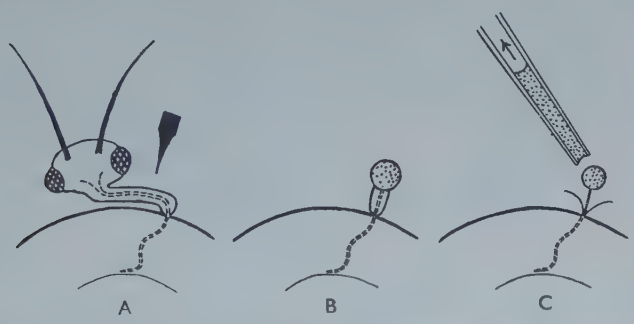

Fig. 1. Sectional views of A, the head of a feeding *T. salignus* with stylet path in host plant and blade about to sever proboscis; B, exudation of stylet-sap from proboscis stump; and C, stylet-sap being collected by a capillary pipette as it exudes from maxillary stylet stump.

brushing the severed tip of the labium from the enclosed stylets, the stylet-sap was observed to exude from the cut end of the 'joined' maxillary stylets; the mandibular stylets generally curling away from the maxillary stylets (Fig. 1 C). This diagram further shows the stylet-sap being collected by means of a glass capillary pipette. For the routine collection of stylet-sap the tip of the capillary pipette was, however, so positioned that the maxillary stylet-stump entered into the lumen of the capillary; the meniscus of stylet-sap at the tip of the capillary forming a viscous seal about the stylet-stump. This position was generally maintained for 5–8 hr. and sometimes for periods of over 24 hr. by embedding the base of the capillary pipette in some modelling clay stuck to a wooden block, or by inserting the pipette into a micro-manipulator placed on the bench. Such arrangements, however, required constant attention as slight vibrations of the bench or draught on the plant's foliage frequently resulted in a displacement of the tip of the pipette and a spilling of the stylet-sap on to the bark.

With the kind co-operation of Mr A. A. Barker a light micro-manipulator was developed which could be attached directly to a willow stem, and hence maintain

the tip of the pipette in position over an exuding stylet-stump for long periods. The instrument, which was constructed by the Cambridge University Engineering Laboratory, is illustrated in Fig. 2. It consists essentially of a clamp which may be attached to stems ranging from approximately 0·3–1 in. in diameter without damaging them. The rod *r*, which carries the pipette holder, can be inserted from either end into a tube *t*, carried by the clamp. When the pipette holder is in position over an aphid colony the rod is secured within the tube by means of the screw *s*. As soon as the proboscis of an aphid has been cut a glass capillary is inserted into the pipette holder to within a few millimetres of the bark, and directed towards the exuding stylet-stump by moving the pipette holder in its ball and socket joint. The final advancement of the pipette towards an exuding stylet-stump is effected by rotating the graduated head *g*. This operates a simple screw adjustment mechanism carried by the clamp, by which the rod and pipette holder may be moved towards or away from the stem.

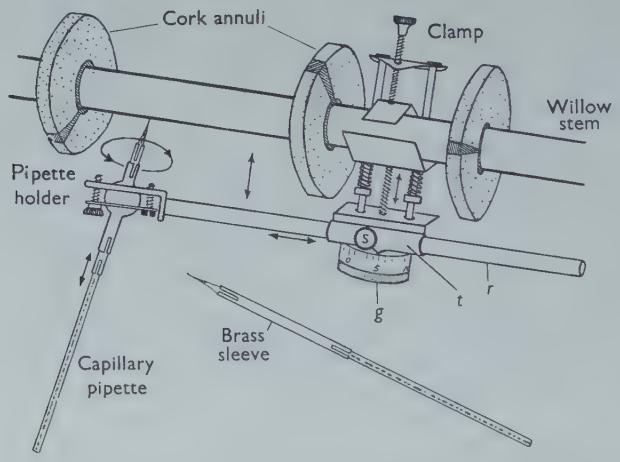

Fig. 2. Instrument used for collecting stylet-sap. It is shown attached by means of the clamp to a willow stem between the cork annuli of adjacent stem cages. The directions in which it may be adjusted are indicated by arrows. For further explanation, see text.

In order to facilitate the rapid replacement of one glass capillary pipette by another during the collection of sap the capillary pipettes were each inserted into a brass sleeve before being inserted into the pipette holder. Sleeves differing in their inner diameter but of constant outer diameter were available so that capillary pipettes of different outer diameters could readily be used in one and the same pipette holder. The volume of stylet-sap collected by a capillary pipette in a certain time was subsequently determined by weighing an equal volume of mercury. The rate of exudation of stylet-sap was thereby also established.

Sap contained in the phloem tissues of pieces of bark, which were stripped from the cambial layer of some *S. triandra* L. stems was squeezed out of the tissues by means of a heavy iron roller.

In order to interrupt the normal translocation of organic solutes in the phloem

of willow stems a girdle of bark 0·25 in. wide and extending to the xylem, was removed from a number 2- to 3-year-old branches of a *S. fragilis* L. tree heavily colonized by *T. salignus*.

Collection of honeydew. Fluid samples of freshly excreted honeydew were collected for analyses by placing a waxed glass plate 1–2 in. under a colony of *T. salignus*. Honeydew droplets falling on this plate were drawn into a glass capillary pipette within a few seconds of their appearance at, and propulsion from, the anus of the aphids.

Chemical methods. The paper chromatographic methods of Consden, Gordon & Martin (1944), Partridge (1948), Williams & Kirby (1948), Crumpler & Dent (1949), and Boggs, Cuendet, Ehrental, Koch & Smith (1950) were used to detect and identify the amino-acids and carbohydrates occurring in stylet-sap and in freshly excreted honeydew. Non-reducing sugars were detected by spraying chromatograms with 5% phosphoric acid in 95% ethanol, and reheating them at 100° C. for 5–10 min. after the reducing sugars had been revealed by the method of Horrocks (1949). Cubic millimetre samples of stylet-sap and of freshly excreted honeydew were applied untreated to Whatman no. 1 filter paper. Some samples were, however, heated in sealed glass-tubing with an equal volume of 6N-HCl for 24 hr. at 100° C., and the acid subsequently removed by desiccation over solid KOH, before the hydrolysate was applied to filter paper. Sap squeezed from the phloem tissues was centrifuged to remove all cell debris before being applied to filter paper.

The total sugar concentrations of stylet-sap and honeydew were estimated by the method of Morris (1948), and the total nitrogen and protein concentrations by the methods of Tompkins & Kirk (1942) and Shaw & Beadle (1949). Nessler's solution, Benedict's uric acid reagent, and the Murexide reaction were used to test for the presence of ammonia and uric acid in freshly excreted honeydew.

Applying the principle of the honeydew clock developed by Smith (1937) honeydew droplets excreted during 24 hr. by colonies of *T. salignus* feeding on intact willow stems were allowed to fall on rotating disks of filter paper. The positions of the honeydew spots on the paper disks were subsequently revealed by spraying the disks with 0·2% ninhydrin in 95% butanol, or with the benzidine reagent of Horrocks (1949), and heating them for 5–10 min. at 100° C. The colour intensity developed by the honeydew spots, furthermore, provided an index of the total amino-acid or sugar concentrations of the honeydew droplets excreted by an aphid colony within a 24 hr. period.

RESULTS

(*a*) *The exudation of stylet-sap*

Stylet-sap exuded from severed stylet-bundles provided they were embedded in turgid willow stems. Observations on the dependence of the exudation on turgor pressure have previously been recorded (Mittler, 1957). Stylet-sap failed to exude

from severed stylet-bundles if they were partially withdrawn from the stem in which they were embedded. This occasionally occurred if the aphids were disturbed before their proboscides were cut. Such failures were, however, entirely eliminated by anaesthetizing the feeding aphids with a gentle stream of carbon dioxide gas before attempting to cut their proboscides.

Although stylet-sap was generally collected from individual severed stylet-bundles for periods of only 5–8 hr. and occasionally for 24 hr., it was frequently shown that stylet-sap continued to exude for a further 2–3 days if the viscous pool of stylet-sap, which formed on the bark about an 'abandoned' severed stylet-bundle, was periodically mopped up with filter paper. On rare occasions stylet-sap ceased to exude after only a few minutes of exudation. Table 1 sets out the rate at which stylet-sap was collected continuously for 72 and 100 hr. from the severed stylet-bundle of a second- and of a third-instar nymph. It may be noted that the rate of exudation diminishes only slightly over these long periods. It has previously been shown that the rate of exudation from the severed stylet-bundles of adult apterous *T. salignus* is greater than that from the severed stylets of the nymphal instars (Mittler, 1957). Stylet-sap for chemical analyses was therefore primarily collected from the severed stylets of adult apterous aphids.

Table 1. *Stylet-sap exudation from the severed stylet bundle of a second and third instar nymph*

Instar	Period of exudation (hr.)	Total volume exuded ($mm.^3$)	Average rate of exudation ($mm.^3$/hr.)	Total sugar concentration (% w/v)
Second	0–25	18·14	0·73	8·60
	25–41	28·89	0·67	—
	41–72	49·34	0·66	8·50
Third	0–18	21·75	1·21	8·51
	18–35	19·35	1·14	8·43
	35–56	18·95	0·91	8·31
	56–76	17·80	0·89	8·38
	76–100	21·10	0·88	8·18

(*b*) *The nitrogen composition*

Aspartic acid, glutamic acid, serine, threonine, alanine, valine, leucine and/or isoleucine, phenyl alanine, asparagine, glutamine and possibly, γ-amino-butyric acid were found in stylet-sap (Mittler, 1953). The greatest concentrations of these amino-acids and amides were found in stylet-sap collected from the severed stylets of aphids which were beginning to colonize willow stems at the termination of the plants' dormancy when bud swelling was prominent; the total nitrogen concentration recorded at this time was 0·2% (w/v).

During bud burst, and subsequently when the first leaves appeared on the willows' branches, the concentration of the amino-acids listed above decreased; the total nitrogen concentration of the stylet-sap falling to 0·12% (w/v). Further

growth of the foliage was accompanied by a further reduction in the concentration of all the amino-acids and amides, as well as in the concentration of total nitrogen. This is in agreement with the results of Lindemann (1948).

During leaf 'maturity' (as used by Kennedy, Ibbotson & Booth (1950) to denote the developmental condition of the foliage between its initial growth and its ultimate senescence) only small amounts of aspartic acid, glutamic acid, glutamine and traces of asparagine were detected in stylet-sap; the total nitrogen concentration was less than 0·03% (w/v) at this time.

During leaf senescence, which was marked by a yellowing of the leaves and the formation of abscission layers at the base of their petioles, the amino-acid composition of stylet-sap resembled that collected from plants in active leaf development; the total nitrogen concentration of the stylet-sap rose to 0·13% (w/v). An abundance of all the amino-acids and amides listed was also detected in stylet-sap 1–2 weeks prior to the death of some of the plants.

No change was detected chromatographically in the number and concentration of the amino-acids and amides in hourly samples of stylet-sap collected during the first 10 hr. of exudation from the cut end of a single stylet-stump. Neither had the number and concentration of the amino-acids and amides changed appreciably after 24 hr.

The amino-acid and amide composition of the honeydew excreted by *T. salignus* feeding on a willow stem was always identical with that of stylet-sap collected at the same time from the same willow stem. Each amino-acid and amide was, however, present at a lower concentration in the honeydew than in the stylet-sap; the relative reduction in the concentration of each amino-acid and amide appeared to be proportionate. The amino-acid and amide composition of the honeydew therefore invariably reflected that of the stylet-sap throughout the seasonal development of the aphids' host plant.

Quantitative total nitrogen determinations showed that *T. salignus* absorb at least 55% of the nitrogenous matter they ingest. The same number and concentration of amino-acids and amide were found to be excreted by adult and by nymphal instars of *T. salignus*. Little or no variation in the intensity of the amino-acid/ninhydrin colour reaction of honeydew droplets which had been deposited on filter paper disks by a colony of *T. salignus* during a 24 hr. period was detected by visual inspection of the paper disks. No appreciable amounts of ammonia, uric acid, proteins, peptides or their breakdown products were qualitatively or quantitatively detected in samples of stylet-sap and honeydew.

The sap squeezed from the phloem tissues of *S. triandra* stems contained the same amino-acids and amides as those found in stylet-sap collected from the same stems. The amino-acid spots on chromatograms of the expressed sap were, however, considerably less intense than those on chromatograms of the stylet-sap, and were partly obscured by peptides. It is interesting to note that the amino-acid composition of the honeydew excreted by coccids, *Eulecanium corni* (Bouché), colonizing the *S. triandra* stems closely resembled that of the honeydew excreted by *T. salignus* feeding on the same plant.

Four to five days after girdling *S. fragilis* branches on which small scattered

colonies of *T. salignus* were feeding, most of the aphids had aggregated to feed immediately above each girdle, were they formed dense colonies 3–4 in. in extent. The nitrogen concentration of the honeydew excreted by these aphids was ten times higher than that excreted by aphids remaining on the branches below the girdles or by those on intact branches of the same tree. Leaves above the girdles rapidly turned yellow and formed abscission layers, as in normal senescence.

(*c*) *The sugar composition*

While the willow trees were bearing leaves sucrose was the only sugar in stylet-sap. The honeydew excreted by *T. salignus*, on the other hand, was found to be composed of roughly equal amounts of sucrose, glucose, fructose and a non-reducing oligosaccharide. The latter was identified as the trisaccharide melezitose; its chromatographic properties being identical to those of an authentic sample of melezitose prepared by the late Dr C. S. Hudson. Glucose and fructose were the only sugars detected in acid hydrolysates of stylet-sap and honeydew.

During the first few days after the aphids had begun to feed on willows whose dormancy had been broken and whose buds were swelling, traces of four non-reducing oligosaccharides were detected in the stylet-sap in addition to sucrose. The honeydew excreted by the aphids at this time contained these oligosaccharides apparently unchanged in quantity and quality, in addition to sucrose, glucose, fructose and melezitose.

The total sugar concentration of stylet-sap obtained from willows kept in the greenhouse was found to lie between 5 and 10 % (w/v) and to be at least 90 % (w/w). The total sugar concentration of stylet-sap obtained from willows which were kept in a room with only a weak illumination and which were colonized by several hundred *T. salignus*, however, gradually fell to almost 1 % (w/v) during 2–3 weeks. No attempt was made to correlate the total sugar concentration of the stylet-sap with the seasonal development of the host plant. The total sugar concentration of the honeydew did not differ by more than 5 % from that of the stylet-sap. The total sugar concentration of the stylet-sap changed only very slightly during its continuous exudation from one and the same stylet-stump (Table 1). No variation in the intensity of the benzidine/sugar colour reaction on filter paper disks of honeydew droplets which had been excreted during a 24 hr. period by a colony of *T. salignus* was detected by visual inspection of the paper disks.

DISCUSSION

Tóth (1946) claimed that the symbiotic micro-organisms of aphids fix atmospheric nitrogen, and that aphids consequently excrete larger amounts of nitrogenous matter than they ingest. If this were the case aphid honeydew should contain nitrogenous materials differing from those ingested. The results of the present investigation indicate, however, that the amino-acids and amides excreted by *T. salignus* are not the products of atmospheric nitrogen fixation, but that each of the amino-acids and amides is ingested in its free form and at a higher concentration

than that at which it is excreted. As these findings did not entirely rule out the possibility that the aphids are fixing atmospheric nitrogen through the agency of their symbionts, a closer examination of this subject was undertaken and will be reported elsewhere (Mittler, in preparation).

A melezitose content of 46·3% (w/w), in the honeydew of *Lachnus roboris* L. was reported by Michel (1942), who did not detect the trisaccharide in phloem sap obtained from the aphid's host plant, and who concluded that the trisaccharide is a by-product of the aphid's digestive processes. The occurrence of considerable amounts of melezitose in the freshly excreted honeydew of *T. salignus*, and its absence in stylet-sap, is further evidence that this trisaccharide is synthesized within the bodies of these lachnids. Gray & Fraenkel (1953) have suggested that the trisaccharide fructomaltose may be expected to arise in the digestive system of any animal that possesses invertase and utilizes sucrose in its diet. Duspiva (1953), Bacon & Dickinson (1957) and Wolf & Ewart (1955) have, furthermore, demonstrated that enzymes, present within the bodies of aphids and coccids, and in their honeydew, are capable of an *in vitro* synthesis of melezitose and other oligosaccharides from sucrose. The fact that considerable amounts of oligosaccharides are present in honeydew excreted by aphids and coccids indicates that these compounds are not merely transitory intermediate products formed during the hydrolysis of dietary sucrose, but that they are purposefully synthesized within the insects' bodies. The significance of these chemical changes has, however, remained obscure. The possibility that intracellular micro-organisms, which occur in the mid-gut epithelium of aphids (Schanderl, Lauff & Becker, 1949), or that enzymes, which occur in phloem sap (Wanner, 1953*b*; Zimmermann, 1954), are involved in these carbohydrate changes should not be overlooked.

Yust & Fulton (1943) claimed that the sap which exudes from the broken ends of the embedded stylet-bundles of *Aonidiella aurantii* (Mask.) is undoubtedly the coccid's food. The results of the present and previous investigation (Mittler, 1957) leave little doubt that stylet-sap is identical with the sap normally ingested by *T. salignus*. The question has been raised, however, whether stylet-sap is identical with the unchanged phloem sieve-tube sap of the aphid's host plant (Duspiva, 1954). As this question is of considerable importance for plant physiological investigations as well as for further aphid nutritional studies, which may make use of the stylet cutting technique, the chemical evidence related to this question will briefly be discussed.

Zweigelt (1914), Davidson (1923) and Bramstädt (1948), have suggested that aphids inject carbohydrases into their host plant to hydrolyse starch or other insoluble carbohydrate matter, and that the breakdown products pass up their stylets. The absence of maltose or other reducing sugar in stylet-sap indicates that its sugar content is not the result of such digestive processes. The almost exclusive occurrence of sucrose in stylet-sap indicates that sucrose is a natural constituent of the phloem sap of willow stems. Sucrose has in fact been shown to predominate in the phloem sap of large numbers of other plants (Wanner, 1953*a*; Ziegler, 1956; Zimmermann, 1957) and has also been shown to occur in the exudate from the

broken stylet-bundles of *Aonidiella aurantii* (Yust & Fulton, 1943). As the sucrose concentration and the rate of exudation of stylet-sap falls off only slowly during 3 days of continuous exudation from a single severed stylet-bundle which is embedded in a turgid willow stem, the exudation cannot be the result of temporary physico-chemical changes which aphids have been supposed to induce within their host plant (Zweigelt, 1914). It has previously been suggested (Mittler, 1957) that the normal turgor pressure of the sieve-tube sap and the large capacity of the phloem is responsible for maintaining the exudation.

The fact that the amino-acid and amide composition of the stylet-sap does not change appreciably during 24 hr. of continuous exudation from a single severed stylet-bundle also shows that proteolytic enzymes, which Bramstädt (1948) has demonstrated in aphid salivary glands, are not hydrolysing plant proteins to give rise to the amino-acids found in stylet-sap. The excretion by *Eulecanium corni* and *T. salignus* of the same amino-acids, suggests that these insects are ingesting sap of the same composition. The results of chromatographic analyses of the sap squeezed from the phloem tissues of willow stems, indicate that the amino-acids and amides found in stylet-sap are in fact present in solution in their free form in the plants' phloem tissues. The fact that the concentrations of amino-acids and amides as well as of sucrose in stylet-sap is maintained over long periods of exudation indicates that water, amino-acids, amides and sucrose pass *en masse* through the sieve-tubes of a willow stem towards the sieve-tube tapped by the stylets.

It is interesting to note that a single *T. salignus* ingesting 10–40 mm.^{3} per day of a phloem sap having a sucrose concentration of 10% (w/v) imposes a sucrose drain of 1–4 mg. per day on its host plant. As 2 mg. of sucrose may be the approximate photosynthetic product of 100 cm.^{2} of leaf per hr. (Spoehr, 1926) the drain imposed on the plant by one aphid per day is equivalent to the amount of carbohydrate a leaf area of 50–200 cm.^{2} may photosynthesize per hr. If photosynthesis were to take place for 10 hr. per day the aphid would ingest the photosynthetic product of 5–20 cm.^{2} of leaf per day.

The seasonal fluctuations in the amino-acid composition and total nitrogen content of the stylet-sap strongly suggest that stylet-sap is identical with the sieve-tube sap of an aphid-free plant. These fluctuations are, moreover, in accordance with plant physiological concepts of the mobilization and conservation of nitrogenous matter by a plant during the growth and senescence of its foliage (Kostytschew, 1931). Kennedy *et al.* (1950) stated that 'it is through a study of the developmental physiology of plants, with special reference to the phloem sap which is the aphids' food, that we may eventually hope to explain aphid distribution.' One may speculate that the recorded fluctuations in the amino-acid composition of stylet-sap are partly responsible for the changing suitability to aphids of plants, or parts of plants, in different developmental conditions.

SUMMARY

1. The aim of this investigation has been to determine the sugar and nitrogen composition of the phloem sieve-tube sap ingested by *Tuberolachnus salignus* (Gmelin) feeding on *Salix acutifolia* stems, and to compare it with that of the honeydew excreted by the aphids.

2. A cage suitable for confining *T. salignus* on the willow stems is described.

3. Details are given of a technique, outlined by Kennedy & Mittler (1953), for collecting the fluid, termed stylet-sap, which exudes from the cut end of severed embedded stylet-bundles.

4. A method is described for collecting honeydew droplets immediately they are excreted by feeding *T. salignus*.

5. The nitrogenous matter ingested by *T. salignus* is in the form of free amino-acids and amides. The same amino-acids and amides are ingested but in greater amounts than they are excreted.

6. The number and concentration of the amino-acids and amides in stylet-sap and honeydew fluctuate with the seasonal development of the host plant.

7. The honeydew contains sucrose, fructose, glucose and melezitose. These sugars are derived from sucrose, the only sugar normally ingested.

8. The evidence for the identity of stylet-sap with the unchanged sieve-tube sap of the host plant is discussed.

REFERENCES

Auclair, J. L. & Maltais, J. B. (1950). Studies on the resistance of plants to aphids by the method of paper partition chromatography. *Canad. Ent.* **82**, 175–6.

Auclair, J. L. & Maltais, J. B. (1952). Occurrence of gamma-amino-butyric acid in extracts of *Pisum sativum* (L.) and in the honeydew of *Myzus circumflexus* (Buck.). *Nature, Lond.*, **170**, 1114–15.

Bacon, J. S. D. & Dickinson, B. (1957). The origin of melezitose; a biochemical relationship between the lime tree (*Tilia* spp.) and an aphis (*Eucallipterus tiliae* L.). *Biochem. J.* **66**, 289–97.

Boggs, L., Cuendet, L. S., Ehrental, I., Koch, R. & Smith, F. (1950). Separation and identification of sugars using paper chromatography. *Nature, Lond.*, **166**, 520–1.

Bramstädt, F. (1948). Über die Verdauungsphysiologie der Aphiden. *Z. Naturf.* **3**, 14–24.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**, 224–32.

Crumpler, H. R. & Dent, C. E. (1949). Distinctive test for alpha-amino-acids in paper chromatography. *Nature, Lond.*, **164**, 441–2.

Davidson, J. (1923). Biological studies of *Aphis rumicis* Linn. The penetration of plant tissues and the source of food supply of aphids. *Ann. Appl. Biol.* **10**, 35–54.

Duspiva, F. (1953). Der Kohlenhydratumsatz im Verdauungstrakt der Rhynchoten, ein Beitrag zum Problem der stofflichen Wechselbeziehung zwischen saugenden Insekten und ihren Wirtspflanzen. *Mitt. biol. ZentAnst., Berl.*, **75**, 82–9.

Duspiva, F. (1954). Weitere Untersuchungen über stoffwechsel-physiologische Beziehungen zwischen Rhynchoten und ihren Wirtspflanzen. *Mitt. biol. ZentAnst., Berl.*, **80**, 155–62.

Evans, A. C. (1938). Physiological relationships between insects and their host plants. I. The effect of the chemical composition of the plant on reproduction and the production of winged forms in *Brevicoryne brassicae* L. (Aphididae). *Ann. Appl. Biol.* **25**, 558–72.

Gray, R. A. (1952). Composition of honeydew excreted by pineapple mealybugs. *Science*, **115**, 129–33.

Gray, H. E. & Fraenkel, G. (1953). Fructomaltose, a recently discovered trisaccharide isolated from honeydew. *Science*, **118**, 304–5.

GRAY, H. E. & FRAENKEL, G. (1954). The carbohydrate components of honeydew. *Physiol. Zoöl.* **27**, 56–65.

HACKMAN, R. H. & TRIKOJUS, V. M. (1952). The composition of the honeydew excreted by Australian coccids of the genus *Ceroplastes*. *Biochem. J.* **51**, 653–6.

HORROCKS, R. H. (1949). Paper partition chromatography of reducing sugars with benzidine as a spraying agent. *Nature, Lond.*, **164**, 444.

KENNEDY, J. S., IBBOTSON, A. & BOOTH, C. O. (1950). The distribution of aphid infestation in relation to leaf age. I. *Myzus persicae* (Sulz.) and *Aphis fabae* Scop. on spindle trees and sugar-beet plants. *Ann. Appl. Biol.* **37**, 651–79.

KENNEDY, J. S. & MITTLER, T. E. (1953). A method for obtaining phloem sap via the mouth-parts of aphids. *Nature, Lond.*, **171**, 528.

KOSTYTSCHEW, S. P. (1931). *Lehrbuch der Pflanzenphysiologie*, Vol. 2. Berlin: Springer.

LINDEMANN, C. (1948). Beitrag zur Ernährungsphysiologie der Blattläuse. *Z. vergl. Physiol.* **31**, 112–33.

MICHEL, E. (1942). Beiträge zur Kenntnis von *Lachnus* (*Pterochlorus*) *roboris* L., einer wichtigen Honigtauerzeugerin an der Eiche. *Z. angew. Ent.* **29**, 243–81.

MITTLER, T. E. (1953). Amino-acids in phloem sap and their excretion by aphids. *Nature, Lond.*, **172**, 207.

MITTLER, T. E. (1957). Studies on the feeding and nutrition of *Tuberolachnus salignus* (Gmelin) (Homoptera, Aphididae). I. The uptake of phloem sap. *J. Exp. Biol.* **34**, 334–41

MORRIS, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthone reagent. *Science*, **107**, 254–5.

PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. *Biochem. J.* **42**, 238–48.

SCHANDERL, H., LAUFF, G. & BECKER, H. (1949). Studien über die Mycetom- und Darmsymbionten der Aphiden. *Z. Naturf.* **4***b*, 50–3.

SHAW, J. & BEADLE, L. C. (1949). A simplified ultra micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples less than 1·0 cu.mm. *J. Exp. Biol.* **26**, 15–23.

SMITH, L. M. (1937). Growth, reproduction, feeding and wing development of the mealy plum aphid, in relation to climatic factors. *J. Agric. Res.* **54**, 345–64.

SPOEHR, H. A. (1926). *Photosynthesis*. New York: The Chemical Catalog Co.

TOMPKINS, E. R. & KIRK, P. L. (1942). Quantitative drop analysis XVI. An improved diffusion method for total nitrogen. *J. Biol. Chem.* **142**, 477–85.

TÓTH, L. (1946). *The Biological Fixation of Atmospheric Nitrogen*. Budapest: Hungarian Museum of Natural History.

WANNER, H. (1953*a*). Die Zusammensetzung des Siebröhrensaftes: Kohlenhydrate. *Ber. Schweiz. Bot. Ges.* **63**, 162–8.

WANNER, H. (1953*b*). Enzyme der Glykolyse im Phloemsaft. *Ber. schweiz. bot. Ges.* **63**, 201–12.

WILLIAMS, R. J. & KIRBY, H. (1948). Paper chromatography using capillary ascent. *Science*, **107**, 481–3.

WOLF, J. P. III & EWART, W. H. (1955). Two carbohydrases occurring in insect-produced honeydew. *Science*, **122**, 973.

YUST, H. R. & FULTON, R. A. (1943). An exudation associated with the feeding location of the California red scale, *Aonidiella aurantii* (Mask). *J. Econ. Ent.* **36**, 346–7.

ZIEGLER, H. (1956). Untersuchungen über die Leitung und Sekretion der Assimilate. *Planta*, **47**, 447–500.

ZIMMERMANN, M. (1954). Über die Sekretion saccharosespaltender Transglukosidasen im pflanzlichen Nectar. *Experientia*, **10**, 145–6.

ZIMMERMANN, M. (1957). Translocation of organic substances in trees. I. The nature of the sugars in the sieve tube exudate of trees. *Plant Physiol.* **32**, 288–91.

ZWEIGELT, F. (1914). Beiträge zur Kenntnis des Saugphänomens der Blattläuse und der Reaktionen der Pflanzenzellen. *Zbl. Bakt.* (Abt. 2) **42**, 265–335.

A CONDUCTIMETRIC METHOD FOR THE ESTIMATION OF SMALL QUANTITIES OF AMMONIA

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INTRODUCTION

Measurements of the ammonia content of fluids are frequently required in biological investigations. The need for these measurements arises not only in studies of the role of ammonia in nitrogen excretion, and in other metabolic processes, but also in connexion with work on many other nitrogenous compounds of biological interest. This is because the analytical procedures for estimating the concentration of many of these substances call for the quantitative analysis of ammonia in the final stage of the technique. The Kjeldahl method for the estimation of total nitrogen can be cited as perhaps the most familiar example of this method of analysis, but similar methods are also widely used for the evaluation of the concentration of urea, amide-N, amino-N and others.

Of the techniques which have been evolved for measuring small quantities of ammonia, that in which the ammonia contained in the sample is liberated as a gas and subsequently recovered in a solution of standard acid is to be preferred because of its ease and high accuracy. Of the various ways in which this has been performed Conway's diffusion method (Conway & Byrne, 1933; Conway, 1933, 1950) has proved the most versatile and adaptable. This method is simple and can be used for measuring quantities of ammonia down to about 1 μg. ammonia-N. Small versions of the diffusion unit, together with ultra-micro titration methods, have pushed the lower limit down to about 0·1 μg. ammonia-N (Glick, 1949; Shaw & Beadle, 1949; Conway, 1950; Kirk, 1950).

In considering the estimation of even smaller quantities of ammonia there is no reason to believe that the present level represents the lower limit of the diffusion method. The limit is set solely by the difficulties of the volumetric procedure for the titration of the absorbing acid. Thus, for the measurement of 1 μg. ammonia-N only 7 μl. of a 0·01 N solution are used for the back titration. To increase the volume of titrating fluid the standard solution can, of course, be diluted, but this leads to a greatly increased difficulty in end-point detection. The difficulties of end-point detection have been partially overcome by the use of a glass electrode assembly for the continuous measurement of the pH of the standard acid solution during titration (Borsook & Dubnoff, 1939), but this arrangement is rather cumbersome and not very suitable for measurements on very small drops.

Although many improvements could, no doubt, be made in titration techniques, it seems that any attempt to reduce the level of estimation to below 0·01 μg. ammonia-N, using the titration method, will come up against grave manipulative difficulties. One solution to the problem is to avoid titration of the standard acid and to use a colorimetric method for the estimation of the absorbed ammonia. This method has been used in micro-ammonia estimations by Borsook (1935) who utilized the blue colour produced by ammonia and an alkaline solution of phenol and hypochlorite. Kirk (1950, p. 284) outlines a procedure using micro-cells and a spectrophotometer, whereby the same colour reaction can be used for ultra-micro measurements with a lower practical limit of 0·01 μg. ammonia-N.

The method described in this paper solves the problem in an entirely different way. It is a conductimetric method which is both simple and reliable, and with the present apparatus allows accurate measurements to be made rapidly down to a level of about 1 mμg. ammonia-N (10^{-9} g.).

PRINCIPLE OF THE METHOD

The fact that the electrical conductance of a standard acid solution decreases as ammonia gas is absorbed forms the principle of the method. The conductance of the acid solution is made up of the separate contributions from hydrogen ions and from the acid anions. Free hydrogen ions are replaced by ammonium ions as ammonia is absorbed by the acid, and the decrease in conductance is due to the difference in mobility between these ions. Thus, for a standard solution of sulphuric acid at 25° C., the mobilities of H^+ and $\frac{1}{2}SO_4^{2-}$ are 350 and 79, respectively, so that the equivalent conductance at infinite dilution is 429 $ohms^{-1}$ $cm.^2$. Ignoring hydrolysis, if the whole of the acid is neutralized by ammonia gas then all free hydrogen ions will be replaced by ammonium ions of mobility 74·5, and the equivalent conductance will fall to 135·5 $ohms^{-1}$ $cm.^2$. The ratio of the conductance of the free acid to the neutralized acid is 2·79. If only 50% of the acid is neutralized then the conductance is the arithmetical mean of the two, and the relationship between conductance and percentage of acid neutralized is a linear one. Conductance measurements are sensitive and can be made with an accuracy at least equal to that of a volumetric procedure. In practice, no manipulations are required other than the transfer of the standard acid solution to the conductivity cell for measurement.

Before turning to the practical details of the method there are certain features of the behaviour of ions in solution which have been overlooked in the simple treatment given above. These are (*a*) that at finite dilutions the equivalent conductances of both acid and salt are somewhat reduced; (*b*) that ion impurities in the water containing the standard acid may make a significant contribution to the measured conductance, and that this may vary with the concentration of free hydrogen ions in the solution; and (*c*) that hydrolysis of the salt produced by neutralization may lead to high values for the conductance. Any of these three factors could affect the linearity of the relationship between measured conductance and the percentage

of the acid neutralized. The influence of each, therefore, will be considered in turn.

(*a*) Table 1 shows some values for the equivalent conductances of ammonium sulphate, and sulphuric acid solutions at given concentrations (from *International Critical Tables*). The conductances decrease in both cases as the concentration is increased, but the ratio of conductances remains constant (within the limits of experimental error) over the range 0–1·0 m-equiv./l. Thus, any dilution of acid within this range can be used. Further, although the concentration of hydrogen ions falls during the absorption of ammonia, it is replaced by an equivalent concentration of ammonium ions and the anion concentration is unaffected—thus the total ionic strength is maintained during absorption and there is no change in equivalent conductance due to this cause.

Table 1. *The equivalent conductances of sulphuric acid and ammonium sulphate solutions*

Concentration (m.-equiv./l.)	Equivalent conductances ($ohms^{-1}$ $cm.^2$)			Conductance ratio
	$\frac{1}{2}(NH_4)_2SO_4$		$\frac{1}{2}H_2SO_4$ 25° C.	
	18° C.	25° C.		
0·1	130	(150·2)	420	2·79
0·2	128	(147·8)	417·9	2·82
0·5	127	(146·3)	413·1	2·82
1·0	124·5	143·4	399·5	2·78
2·0	122	141·5	390·5	2·76

The figures are taken from *International Critical Tables* and those in brackets are calculated from the temperature coefficients.

(*b*) The chief source of ion impurity in the water is carbon dioxide absorbed from the atmosphere. Distilled water can be specially prepared (for example, by passing the first batch of water through a mixed ion exchange resin) with a conductance which is insignificant compared with that of the standard acid but, unless special precautions are taken, this rapidly increases to an equilibrium value due to the uptake of atmospheric carbon dioxide. The contribution of dissolved carbon dioxide, in equilibrium with the atmosphere, to the conductance of the water can easily be calculated from its concentration in the water ($1{\cdot}7 \times 10^{-5}$ M; see Conway, 1950, p. 317), its dissociation constant ($3{\cdot}0 \times 10^{-7}$) and the equivalent conductance (399·5). The specific conductance of the dissolved CO^2 is $0{\cdot}85 \times 10^{-6}$ $ohms^{-1}/cm$. In acid solution the ionization of the carbon dioxide will be suppressed and hence its conductance greatly reduced. In Table 2 the specific conductances of some standard, CO^2-free sulphuric acid solutions are given together with similar figures for solutions partially and completely neutralized with ammonia. By the side of each is a value for the additional specific conductance due to dissolved CO^2, in equilibrium with the atmosphere, calculated in each case with due regard to the pH of the acid solution. The table shows that for an acid concentration of 1 m-equiv./l. the conductance due to CO^2 is for all practical purposes negligible. An acid solution of

0·1 m-equiv./l. can also be used providing that it does not absorb more than about 90% of ammonia necessary for complete neutralization. This is the practical lower limit for the dilution of the standard acid. At concentrations below this the conductance due to CO^2 is marked, and such solutions cannot be used unless special precautions are taken to use CO^2-free distilled water and to work in a CO^2-free atmosphere.

Table 2. *The specific conductances (ohms^{-1}/cm. $\times 10^{-6}$) of CO_2-free mixtures of sulphuric acid and ammonium sulphate solutions of the same concentration together with values for the additional specific conductance due to dissolved carbon dioxide.*

Ratio $\frac{H_2SO_4}{H_2SO_4+(NH_4)_2SO_4}$ (%)	Concentration of H_2SO_4 and $(NH_4)_2SO_4$ mixtures (m-equiv. l.)					
	1·0	CO_2 conduct-ance	0·1	CO_2 conduct-ance	0·01	CO_2 conduct-ance
100	399·5	0·002	42·0	0·02	4·29	0·20
70	322·7	0·004	33·9	0·04	3·46	0·27
40	235·8	0·006	25·8	0·06	2·63	0·41
10	169·0	0·02	17·7	0·20	1·81	0·70
0	143·4	0·85	15·0	0·85	1·53	0·85

(*c*) The effect of hydrolysis of the ammonium salt on the measured conductance can be readily calculated. At a salt concentration of 0·1 m-equiv./l. the specific conductance is increased from 15 to $15{\cdot}07 \times 10^{-6}$ ohms^{-1} cm. in the absence of dissolved CO_2. If CO_2 is present, as is generally the case, then the hydrolysis is greatly reduced by mutual interaction and its effect on the total conductance can be safely ignored.

The principles outlined above were tested. Standard solutions of sulphuric acid and ammonium sulphate were prepared in the same concentrations. The solutions were mixed in known proportions to simulate acid solutions in which known amounts of ammonia had been absorbed. The conductance of each mixture was measured in a small conductivity cell (this cell had a volume of 10 μl. and its construction is described below) and plotted against the proportion of H^+ to total cations in the mixture. The cell constant was measured in the usual way with standard potassium chloride solutions. The results for two different concentration (1·0 and 0·1 m-equiv./l.) are shown in Fig. 1. For the 1·0 m-equiv. l. solutions very good linearity was found and the ratio of conductances of acid and salt (2·75) agrees well with that predicted from theory (2·78; see Table 1). In the more dilute solutions the relationship is also linear until the acid concentration falls to about 0·02 m-equiv./l.; below this the effect of CO_2 increases the conductance in the manner predicted above. The slope of the straight part of the line corresponds to an acid/salt ratio of 2·78; again in excellent agreement with the predicted value of 2·79. In both cases the specific conductances approximate to the values expected from Table 2.

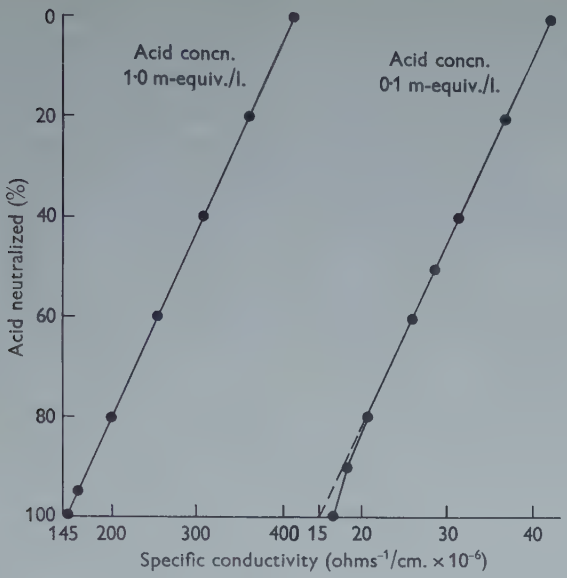

Fig. 1. The relation between specific conductivity and percentage of acid neutralized with ammonia in two standard sulphuric acid solutions.

PRACTICAL DETAILS

Ammonia liberated from the sample by alkali in a small diffusion chamber is absorbed in a small drop of standard acid. At the end of the diffusion period the acid drop is transferred to a small conductivity cell maintained at constant temperature and made up to a mark with distilled water. The conductance of the solution is measured and the percentage of the acid neutralized is calculated by reference to a calibration curve.

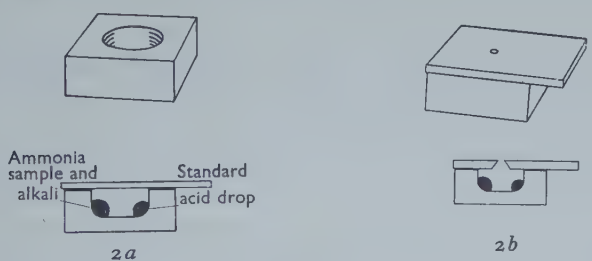

Fig. 2. The diffusion chamber. (*a*) The normal chamber together with a sectional view to show the position of the reagent drops. (*b*) The modified chamber for use at low ammonia levels together with a sectional view.

(*a*) *The diffusion procedure*

The diffusion apparatus (Fig. 2*a*) consists of a brass chamber, approximately 8 mm. in internal diameter and 3·5 mm. deep, and a glass lid which projects in one direction beyond the chamber, the projection forming a handle for ease of manipulation. The chamber is coated internally with paraffin wax.

The sample drop and the drop of strong alkali (3–5 μl. of half-saturated potassium metaborate solution) are placed close to each other in the bottom of the chamber on one side, the standard acid drop (1–3 μl. of a standard sulphuric acid solution, e.g. 0·001 N), from an ultra-micro pipette, is placed on the other. Next, the lid is sealed in position by means of a paraffin wax/liquid paraffin fixative (Conway, 1950, p. 93), and the alkali and sample drops mixed by tapping the chamber. The chamber is now left until complete absorption of ammonia has occurred.

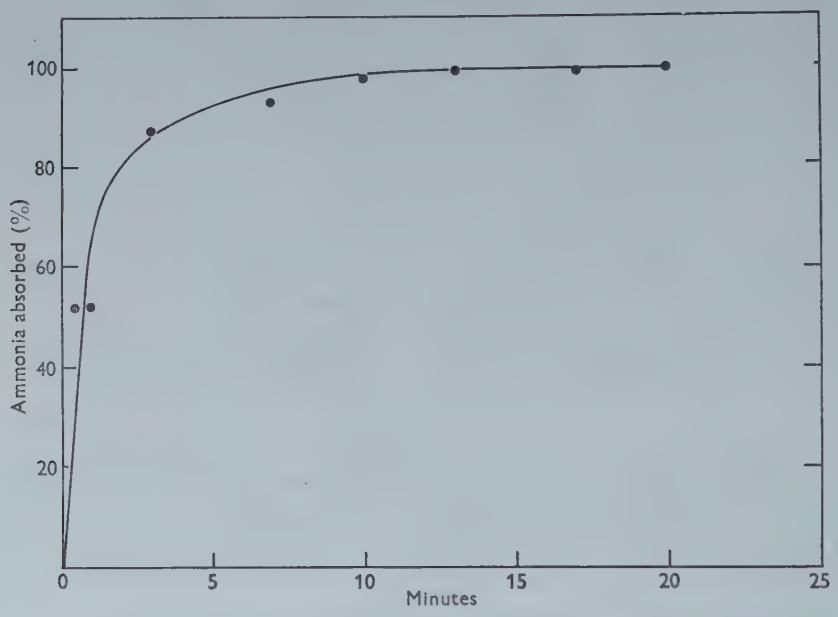

Fig. 3. The time course for the diffusion of ammonia (8·3 mμg. ammonia-N) into 1·48 μl. of 0·5 m.-equiv./l. H_2SO_4.

The progress of a typical diffusion at 20° C. is shown in Fig. 3. Diffusion is quite rapid. In the case illustrated it is complete in 20 min. For most conditions 30 min. can be regarded as a suitable period of time for diffusion.

In many laboratories ammonia may be present in the air in quantities sufficient to create an appreciable error in the measurement of the smallest quantities of ammonia (1–10 mμg.). The presence of extraneous ammonia can be revealed if a normal diffusion is performed without an ammonia-containing sample; a fall in the conductance of the standard acid drop indicates the uptake of ammonia. If detectable quantities of ammonia are present it is necessary to employ a slightly modified version of the diffusion apparatus (Fig. 2*b*), the interior of which can be cleared of ammonia before commencing an actual diffusion. The chamber remains unchanged, but the lid, now made of Perspex, has a small hole drilled in the centre. Prior to diffusion, alkali and a drop of standard acid are introduced into the chamber, and the lid then sealed into position (an alternative, which has been found very satisfactory in practice, is to seal the lid into position with wax, by applying to the

chamber when the coating wax is still molten, and later to introduce all solutions through the hole in the lid; the wax seal is completely effective and easily broken when necessary). The small hole in the lid is temporarily covered with a microscope cover-slip. The apparatus is left for a period of 30 min. during which time any extraneous ammonia in the chamber is absorbed by the standard acid drop. At the end of this period the acid drop is removed by inserting a pipette through the small hole, and the sample and another drop of standard acid introduced. Between the introduction or removal of solutions the cover-slip remains over the aperture. Finally, the cover slip is removed, the small hole immediately sealed with a small blob of hot wax, and the sample and alkali drops mixed. The diffusion process now proceeds in the normal way.

(*b*) *Conductance measurement*

The conductivity cell is made in Perspex and consists of three pieces bolted together in the form of a sandwich, the middle piece being drilled to make the cell (see Fig. 4). On the inner faces of the outer pieces small circles of bright platinum foil (cleaned in chromic acid and very well washed) just larger than the diameter of the cell are cemented. These form the two electrodes and thin strips from them are brought out through the lower bolt holes, the bolts of which are used as the electrode terminals. A narrow hole in the edge of the middle piece and at right angles to the cell serves as a neck for filling the cell. The cell is filled to a mark about half-way up the neck. The edges of the block are polished so that the cell and its contents can be viewed from front and back and also from the sides. The dimensions of the cell depend on the range of ammonia estimations being made. Thus, in the lowest range the cell diameter is $\frac{1}{16}$ in. and length also $\frac{1}{16}$ in. giving a volume, including the neck region, of about 5 μl. By increasing the diameter of the cell, or by increasing the thickness of the Perspex forming the middle piece, greater volumes can be accommodated.

When in use the block containing the cell is bolted to a long narrow piece of Perspex which serves as a handle for inserting the cell into a tube immersed in a thermostatically controlled water-bath.

The cell electrodes can be connected to any suitable conductivity bridge—a Mullard type E7566 can be used if it is provided with additional standard resistances so that the readings can always be brought to the middle part of the potentiometer scale.

The cell is thoroughly washed with distilled water before introducing the standard for estimation. Transfer of the standard is effected by means of a capillary pipette of capacity approximately four times greater than that of the cell. Before sucking in the standard, the pipette is half-filled with distilled water. The point of the pipette is introduced down the neck to the bottom of the cell, and then the acid sample is blown out together with sufficient water to fill the cell on the withdrawal of the pipette. The distilled water remaining in the pipette serves as a barrier preventing exhaled CO_2 from contaminating the cell contents.

If the sample volume is small compared with the volume of the neck the cell need

never be emptied of fluid; an advantage if it shows a propensity to trap small air bubbles beneath the shoulders against the surface of the electrodes. The cell is flushed with water and drained to the base of the neck only.

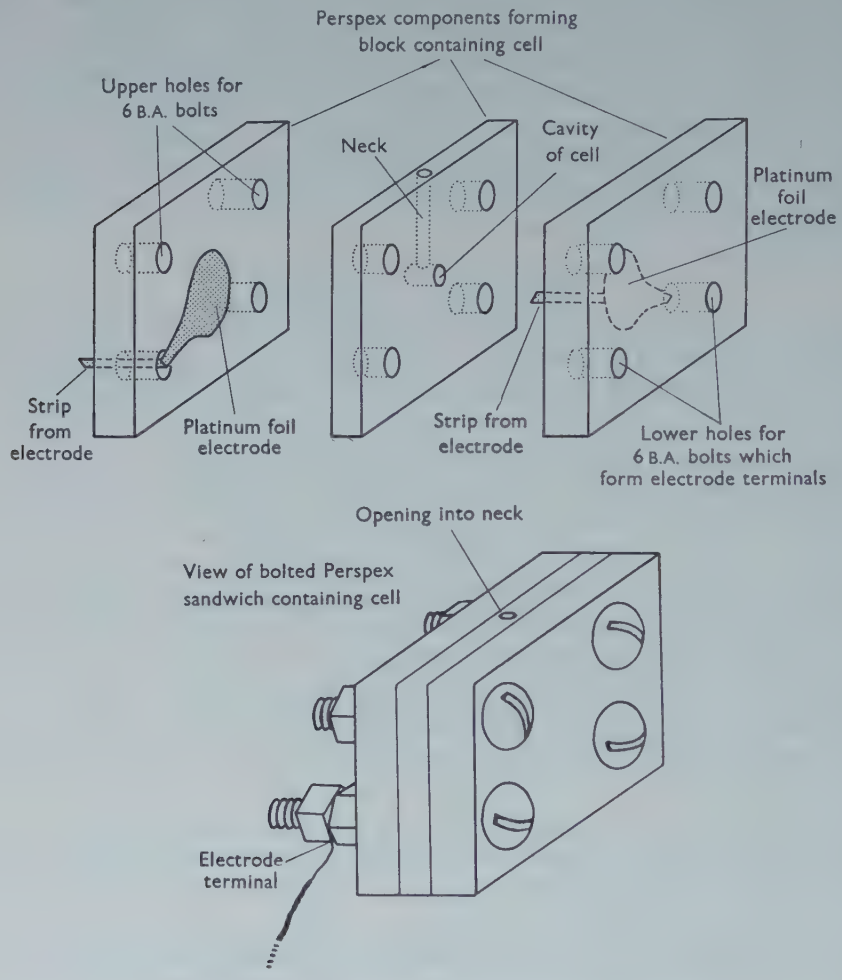

Fig. 4. Details of construction of a conductivity cell.

The cell, when filled, is lowered by means of the Perspex handle to the bottom of a Perspex (or thin glass) tube which is immersed, almost to the top, in a temperature-controlled water-bath. (Any delay experienced waiting for temperature equilibrium can be avoided by washing and filling the cell *in situ*, but this calls for greater care.)

The fluid in the cell is now thoroughly stirred for a period of $\frac{1}{2}$ min. The stirrer consists of a thin polythene rod attached to a narrow glass rod which acts as a handle. Stirring is accomplished by introducing the polythene rod through the neck of the cell and spinning the glass handle between the forefinger and thumb of one hand.

When a cell is used for the first time a calibration curve must be produced. For this purpose a series of mixtures are prepared from standard sulphuric acid and ammonium sulphate solutions of the same concentration. The primary standards, 0·1 N-H_2SO_4 and 0·1 N-$(NH_4)_2SO_4$, are stored in polythene bottles. These primary standards are mixed in different proportions and suitably diluted when required for the preparation of the calibration curve. The conductance of each mixture is determined and the results obtained plotted against the proportion of hydrogen ions to total cations.

With new cells the first readings on acid solutions are sometimes too low so that the cell should be washed out several times with the solution until a consistent reading is obtained. Low readings may also occur if a cell has been left for a period without use. Prior to a diffusion analysis, therefore, it is advisable to charge the cell several times with a volume and concentration of standard acid identical with that which will be employed in the diffusion chamber until a constant value for the conductance is obtained. This value should be checked against the value obtained in the original calibration.

RESULTS

Over a hundred measurements on standard ammonium sulphate solutions ranging in content from 2 mμg. to 12 μg. N are summarized in Table 3. Conductivity cells of different sizes were used for the various ranges. A standard commercial cell, 2 ml. in volume, was found to be satisfactory for estimating quantities of ammonia ranging from 0·5 to 12 μg. N. For all other ranges Perspex cells were used. The recovery of ammonia was invariably complete although sometimes a little in excess, particularly with respect to the smallest quantities estimated in certain ranges.

Measurements were made on samples containing respectively 20 and 80% of that quantity of ammonia required to neutralize the standard acid fully. These quantities approach the limits of the range. From Table 3 it is evident that measurements can

Table 3. *Ammonium sulphate solutions*

Range monia-N (μg.)	Ammonia-N expected (μg.)	Standard acid neutralized (%)	Ammonia-N found (μg.)	% recovery	No. of measure-ments	Standard deviation (μg. ammonia-N)	Standard deviation (%)
·5–12	11·92	80	11·96	100·3	11	0·11	0·92
	2·98	20	3·15	105·7	18	0·09	2·86
·5–2·5	2·38	80	2·40	100·8	7	0·02	0·83
	0·60	20	0·60	100·0	7	0·01	1·67
mμg.)	(mμg.)		(mμg.)			(mμg. ammonia-N)	
30–150	143·3	80	143	99·8	9	0·6	0·42
	35·8	20	36	100·6	10	1·2	3·33
7–32	30·24	80	30·4	100·6	19	0·4	1·32
	30·24	80	30·3	100·1	8	0·4	1·32
	7·56	20	7·8	103·2	10	0·3	3·85
	7·56	20	8·0	105·8	8	0·4	5·0
1–9	8·28	80	8·24	99·5	8	0·06	0·73
	2·07	20	2·14	103·0	9	0·08	3·74

be made in any range with an error $< \pm 4\%$ (standard deviation); with an error $< \pm 1\%$ provided that 80% of the standard acid is neutralized by ammonia. The percentage error of measurements in the range 7–32 mμg. ammonia-N is somewhat higher but this, no doubt, can be reduced. Mid-range quantities of ammonia, neutralizing 50% of the standard acid, can be expected to show a standard deviation, of ± 1–2% in all ranges. The method can, therefore, measure quantities down to 5 mμg. ammonia-N with an error $< \pm 2\%$, provided that at least 50% of the acid is neutralized during diffusion. Measurements on samples containing 1 mμg. ammonia-N can be expected to show a standard deviation of ± 6–8%.

Table 4. *Biological fluids*

Species	Material	Vol. of sample (μl.)	No. of measurements	Ammonia-N (mμg.)	Standard deviation (mμg.)	Conc. of ammonia-N (mg. N/100 ml.)
Aeshna cyanea, nymph	Haemolymph	0·90	6	1·5	0·07	0·17
		0·58	6	3·1	0·07	0·53
		0·58	7	1·3	0·07	0·22
Sialis lutaria, larva	Haemolymph	2·22	3	18·0	—	0·81
		2·22	3	5·6	—	0·25
	Rectal fluid	0·10	3	132	—	132
		0·06	3	66	—	110
Carcinus maenas, adult	Haemolymph	2·99	22 (from 6 animals)	—	—	0·32
	Single muscle fibres	—	16 (from 4 animals)	—	—	3·93

Some measurements on biological materials are listed in Table 4. The measurements on haemolymph and excretory fluid hardly require comment, except that there was no evidence of ammonia production in the shed haemolymph of either *Aeshna*, *Sialis* or *Carcinus*, and that little more than 0·001 μl. would have sufficed for a single estimation on the rectal fluid of *Sialis*, in which the concentration of ammonia frequently exceeds 100 mg. N/100 ml. The measurements obtained on single muscle fibres from *Carcinus maenas* illustrate the utility of the method in determining the ammonia content of large cells. The dissected fibre, placed directly in the diffusion chamber, is disrupted by the strong alkali and the free ammonia liberated is collected in the usual manner.

Sufficient estimations were made on the blood of *Aeshna* nymphs to enable standard deviations to be calculated. The standard deviations obtained were of the same order as standard deviations calculated from measurements on solutions of ammonium sulphate.

No attempt has been made to reduce the level of ammonia estimation below 1 mμg. N, although there seems no doubt that this can be done if necessary. The smallest conductivity cell employed in this investigation had a volume of 5 μl., but smaller cells can be made. Bayliss & Walker (1930) used for their studies on the

amphibian glomerular fluid a cell with a volume as small as 0·5 μl. Although their cell had a number of defects the principle of using the cut-ends of pieces of platinum wire as electrodes could, no doubt, be used to make successful micro-cells.

SUMMARY

1. A method is described for the estimation of small quantities of ammonia down to 0·001 μg. N.

2. The principle of the method is that ammonia is liberated from the sample and recovered in a solution of standard acid by Conway's diffusion method; the electrical conductance of the acid falls as ammonia is absorbed, due to the difference in the mobility of hydrogen and ammonium ions.

3. The results of estimations on standard ammonium sulphate solutions and biological fluids are tabulated. The method can deal with quantities down to 0·005 μg. ammonia-N with an error $< \pm 2\%$ (standard deviation), 0·001 μg. ammonia-N with an error ± 6–8%.

4. An account is given of the construction of small diffusion chambers and small conductivity cells.

REFERENCES

Bayliss, L. E. & Walker, A. M. (1930). The electrical conductivity of glomerular urine from the frog and from *Necturus*. *J. Biol. Chem.* **87**, 523–40.

Borsook, H. (1935). Micromethods for determination of ammonia, urea, total nitrogen, uric acid, creatinine (and creatine), and allantoin. *J. Biol. Chem.* **110**, 481–93.

Borsook, H. & Dubnoff, J. W. (1939). Methods for the determination of submicro quantities of total nitrogen, ammonia, amino nitrogen, amides, peptides, adenylic acid and nitrates. *J. Biol. Chem.* **131**, 163–76.

Conway, E. J. (1933). An absorption apparatus for the micro-determination of certain volatile substances. II. The determination of urea and ammonia in body fluids. *Biochem. J.* **27**, 430–4.

Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*. 3rd revised ed. London: Crosby Lockwood.

Conway, E. J. & Byrne, A. (1933). An absorption apparatus for the micro-determination of certain volatile substances. I. The micro-determination of ammonia. *Biochem. J.* **27**, 419–29.

Glick, D. (1949). *Techniques of Histo- and Cytochemistry*. New York: Interscience.

Kirk, P. L. (1950). *Quantitative Ultramicroanalysis*. New York: John Wiley and Sons.

Shaw, J. & Beadle, L. C. (1949). A simplified ultra-micro Kjeldahl method for the estimation of protein and total nitrogen in fluid samples of less than 1·0 μl. *J. Exp. Biol.* **26**, 15–23.

THE MOVEMENT OF THE SPERMATOZOA OF THE BULL

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(With Plates 2–4)

The rate at which the spermatozoa of a sea-urchin propel themselves can be expressed, with surprising accuracy, in terms of the form and frequency of the bending waves generated by the tail (Gray, 1955; Gray & Hancock, 1955). The object of this paper is to consider how far this conclusion is applicable to other types of flagellated spermatozoa; those of the bull with their relatively larger heads and thicker tails provide a useful basis of comparison and contrast.

Under continuous dark-ground illumination an active spermatozoon of the bull exhibits a well-defined optical envelope, usually triangular in outline, with an apex either at the posterior or at the anterior end of the middle piece. Attempts to correlate the form of the envelope with the form of the waves passing over the tail by stroboscopic illumination proved to be much less satisfactory than those based on direct photography. The records shown in Pl. 2 were obtained by means of the equipment described by Brown & Popple (1955), so arranged as to give a known number (three to eight) successive electronic exposures on a stationary film at intervals of $\frac{1}{80}$ sec. In photographs 2–5 and 12–14 the number of flashes was sufficient to cover at least half of a complete contractile cycle, and the outline of these figures gives the general form of the envelope which would have been seen under continuous illumination. Pl. 2 illustrates three types of movements. (i) Those in which transverse movement is restricted to the tail (e.g. photographs 1–5). (ii) Those in which both tail and middle piece show transverse movement, although the head does not (e.g. photograph 6). (iii) Those in which head, middle piece and tail all show lateral displacement (e.g. photographs 8–14). It seems likely that these differences are largely due to differences in the degree to which lateral movements of the head and middle piece were restrained by contact with the slide; in the absence of such restraint photographs 1–7 would approximate to photographs 8–14. This conclusion is supported by the records shown in Pl. 3 which were obtained on a moving film using flashes at $\frac{1}{66}$ sec. intervals; lateral oscillation of the head is clearly detectable.

Photographs similar to those shown in Pls. 2 and 3 reveal five features which are relevant to an analysis of the propulsive mechanism of the tail.

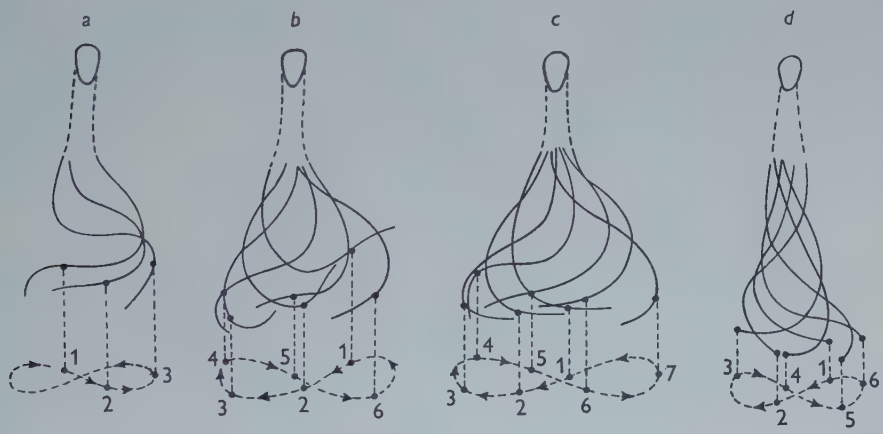
(i) The maximum extent to which an element of the tail bends during its contractile cycle is not the same for all elements. The nearer the element lies towards

the tip of the tail the greater is the amount of bending; this is clearly seen by following the dot (●) marked on the left side of the tail in Pl. 3, photographs 2–8.

(ii) The phase difference between successive elements varies along the length of the tail. Pl. 3 shows that the length of tail between two points on the tail (●, +) which differ in their phase by one-half of a complete cycle is greater at the proximal end of the tail than at the distal end; in other words, the length and speed of propagation of the 'wave' decrease as its crest moves backwards along the tail.

(iii) The amplitude of transverse movement—relative to the head—increases progressively along the tail towards the distal end (Pl. 2, photographs 1–5).

(iv) An element lying towards the distal end of the tail executes a figure-of-eight motion relative to the head; at certain phases of its motion such an element moves backwards relative to the head (Text-fig. 1).

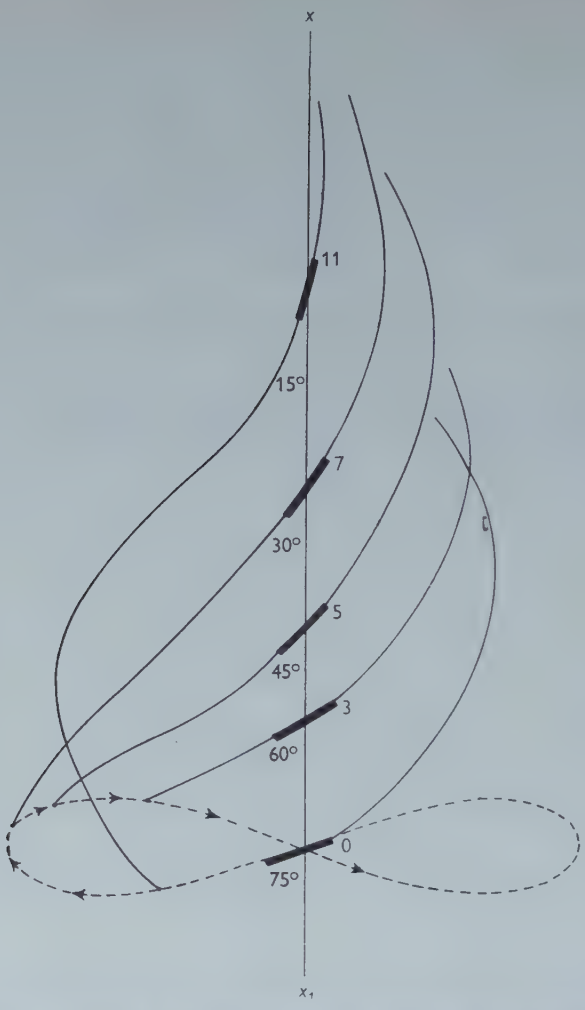


Text-fig. 1. Diagram illustrating the figure-of-eight paths, relative to the head, followed by points lying towards the distal end of the tail (see Pl. 2, photographs 1–4). In Text-fig. 1 *a–c* the point is approximately 10 μ from the tip of the tail; in Text-fig. 1 *d* it is at the tip of the tail. The numbers indicate successive stages of the bending cycle.

(v) The angle at which the surface of an element crosses the axis of propulsion increases progressively the nearer the element lies towards the distal end of the tail (Text-fig. 2).

All these characteristic features of the movement of the tail indicate departures from the conditions which form the basis of the argument applied to the sea-urchin (Gray and Hancock 1955); their theoretical significance will be discussed later in this paper.

Text-fig. 3 shows the effect of changes in the maximum degree of bending and in the phase difference between adjacent elements upon the wave-length and amplitude of the resultant waves.

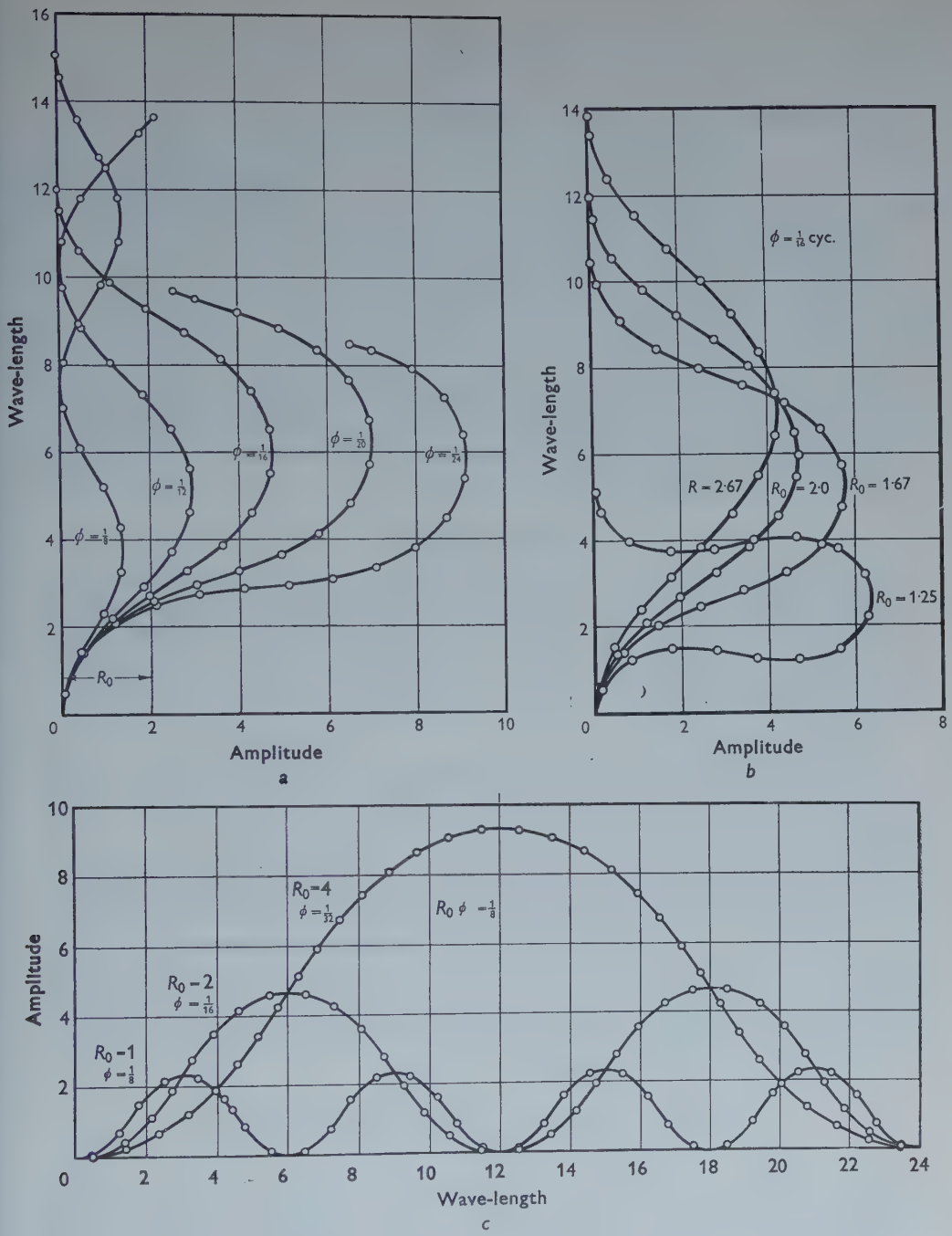


Text-fig. 2. Diagram illustrating the variation of the average angle of inclination (θ) between elements, situated at various positions along the length of the tail, and the axis of propulsion (xx_1). The arabic numerals indicate the relative distance of an element from the tip of the tail.

Frequency of bending cycles

As determined photographically the average frequency of the bending cycles exhibited by thirty-one cells in the samples was 9·1/sec. at 37° C., but as shown in Table 1 the range of variation was very considerable, not only between the two samples of sperm but also among the individuals of the same suspension.

The order of frequency recorded for these samples of bull's spermatozoa is much less than that found for the sea-urchin (33–40/sec. at 17° C.; Gray, 1955) in spite of the fact that the environmental temperature was much greater. The average translatory speeds of the bull's spermatozoa were rather low (see below); other suspensions might have yielded higher frequencies. During each complete



Text-fig. 3. Relationship of the form of a wave to the maximum degree of bending and the phase difference between adjacent segments. The maximum degree of bending is defined by the reciprocal of the minimum radius of curvature (R_0) and the phase difference per unit length as a fraction (ϕ) of one complete cycle.

(3*a*). In all cases $R_0 = 2$; ϕ varies from $\frac{1}{8}$ to $\frac{1}{24}$ cyc. Both wave-length and amplitude decrease with increase of ϕ.

(3*b*). In all cases $\phi = \frac{1}{16}$; R varies from 1·25 to 2·67. An increase of R_0 causes an increase of wave-length, but a decrease in amplitude.

(3*c*). If $R_0\phi$ is constant, the general form of the wave remains the same but its absolute size changes.

Table 1

Suspension	Frequency of waves per sec.														Total cells	Mean frequency	Mean translatory velocity (μ/sec.)	Mean translatory distance per cycle (μ)
	3	4	5	6	7	8	9	10	11	12	13	14	15	16				
A	0	1	3	1	4	3	3	3	0	0	0	0	0	0	18	7·5	79	10·5
B	—	—	—	—	0	2	3	0	3	1	0	2	2	0	13	11·3	66	6·0
Total	—	1	3	1	4	5	6	3	3	1	0	2	2	—	31	9·1	73	8·0

contractile cycle a spermatozoon of a sea-urchin moves forward a relatively constant distance of 5·5 μ; those of the bulls used for Table 1 varied, but moved forward an average distance of 8·3 μ. For a wave frequency of 15/sec., the speed of translatory movement would be approximately 120 μ/sec.

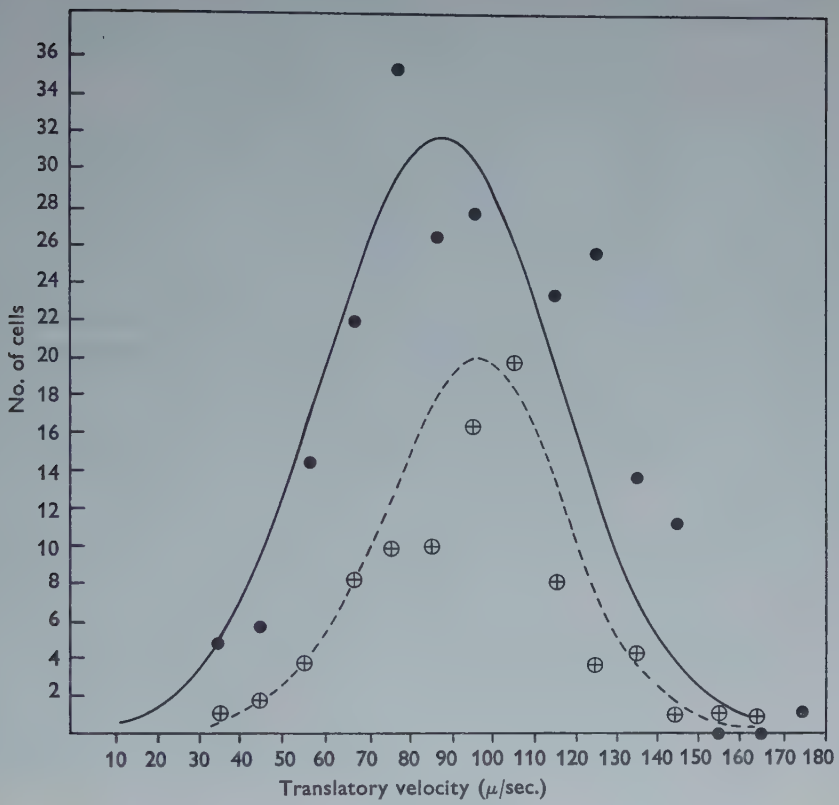
Translatory velocity

Correlated with a relatively low frequency of propagated bending cycles, the spermatozoa of the bull exhibit a relatively low speed of progression through the water. As shown in Table 2, the average velocity of 235 cells derived from eleven samples was 94 μ/sec., but as shown in Table 2 and by Text-fig. 4 the spread of variation between different samples and between individuals in a single sample was very considerable.

An average translatory speed of 94 μ/sec. appears to be substantially lower than that (123 μ) observed by Rothschild (1953*a*). The two figures are, however, not comparable, since the latter records the length of the undulatory track of the head, whereas Table 2 records the displacement along the axis of progression. An examination of Lord Rothschild's films showed that the average ratio of these two measurements is 1·4, and consequently the average speed recorded in this paper represents 131 μ/sec. when measured along the track of the head. As shown in Text-fig. 4 the spread of translatory speed observed in the present work was similar to that recorded by Rothschild. So far as could be determined from a single sample (Text-fig. 4) the absence of the head does not substantially increase the propulsive speed (see Gray & Hancock, 1955).

Movements of the spermatozoon about its median longitudinal axis

The cells figured in Pls. 2 and 3 were moving in close proximity to the surface of the slide and they present one of the flat surfaces of their heads towards the observer during the whole of the contractile cycle. When swimming freely in a relatively deep drop, the head often presents to the observer a broad surface and a narrow surface alternately, thus producing, under dark ground illumination, the well-known 'flashing' effect; the head either 'rocks' or 'rolls' about its median longitudinal axis. During the phases at which the head is seen 'edge on', the optical envelope of the tail approximates to a brightly illuminated line (Pl. 4*a*,



Text-fig. 4. Diagram showing variation in propulsive speed. Normal cells, ●; headless cells, ⊕. The curves have the same characteristics as those of Rothschild (1953*a*) adjusted for the number of cells involved.

Table 2

Suspension	No. of cells recorded	Mean translatory velocity (μ/sec.)
A (*c*)	31	111
B (*h*)	14	118
C (*h*)	21	115
D (*h*)	18	120
E (*h*)	7	129
F (*c*)	24	80
G (*c*)	56	72
H (*h*)	25	94
I (*h*)	15	99
K (*c*)	14	66
L (*c*)	10	65
Total	235	94

(*c*) Determined cinematographically, (*h*) determined by length of head-track per sec.

photograph 3), thus showing that most, if not all, of the elements of the tail execute their transverse movements in a plane coincident with that of the median transverse axis of the head. In thirty-one cells—taken from the same suspension—the frequency of 'flash' varied from 4 to 11/sec. and gave an average value of 8·2/sec.; this is of the same order as that of the average wave frequency.

The tendency to roll about the longitudinal axis does not depend on the presence of the head (see Pl. 4(*b*)); the 'flashing' of the head must be due to the activity of the tail. An exact correlation between the frequency of flashing and the frequency of the bending waves is shown in Pl. 4(*c*), where the head 'flashed' when a region of maximum curvature passed over the distal end of the tail. This condition would arise if the passage of a bending wave along the tail were accompanied by a torsional wave, or if the plane of vibration of the distal region of the tail differed from that of the proximal regions; in the first case flashing could occur if the bending wave were suppressed; in the second case, the distal end of the tail would, at the moment of flash, be deflected laterally as in Pl. 4(*d*). Most, if not all, of the present observations suggest that the tail is slightly twisted when a wave of curvature passes over its distal region.

In some cases the head flashes when its own longitudinal axis lies along the axis of progression, and a dark-ground photograph yields a series of flashes aligned along this axis (see Rothschild, 1953*b*). In other cases the flashes may be inclined at an angle to this axis to form a series of oblique parallel lines, or a more complex herring-bone pattern. If, as seems probable, a flash depends on the forces exerted against the water by the distal elements of the tail, the onset of a flash would depend on the difference in phase between the lateral displacements of the head and of the end of the tail.

DISCUSSION

The reaction exerted by the water against any short element of a flagellum depends on three main variables. (i) The angle (θ) which its surface subtends with the main axis of forward propulsion. (ii) The velocity (V_y) at which the element travels transversely to this axis. (iii) The velocity (V_x) at which it travels along this axis. The reaction from the water has two components, one (dF) acting along the axis of propulsion and the other (dT) acting transversely. The relative magnitude of both these forces can be determined by methods similar to those applied to the propulsive force of a sea-urchin's spermatozoon (Gray & Hancock, 1955).

Initially, it is convenient to consider the distal element of the tail of a bull's spermatozoon whose head is stationary relative to the surrounding waters as in Text-fig. 5; the motion of the element relative to the water is then the same as its motion relative to the head. Such an element has a transverse velocity (V_y) and a longitudinal velocity (V_h) relative to the head; the latter component alters its sign twice during each complete cycle, and will be regarded as positive when directed away from the head, i.e. in the same direction as that in which the waves of changing curvature pass along the tail. The displacements of the element due to V_h and V_y are equivalent to a tangential displacement V_t and a displacement V_n normal to the element's surface. If the resistance to flow along the surface is $C_L V_t$, whilst that

normal to the surface is $2C_L V_n$ (see Hancock, 1953) the total propulsive thrust (dF) and total transverse force (dT) are defined by equations (i) and (ii)

$$dF = C_L[V_y \sin\theta \cos\theta + V_h(\sin^2\theta + 1)]\, ds, \quad \text{(i)}$$

$$dT = C_L[V_y(\cos^2\theta + 1) + V_h \sin\theta \cos\theta]\, ds. \quad \text{(ii)}$$

If $\sin\theta\cos\theta = a$, $\sin^2\theta + 1 = b$ and $\cos^2\theta + 1 = c$

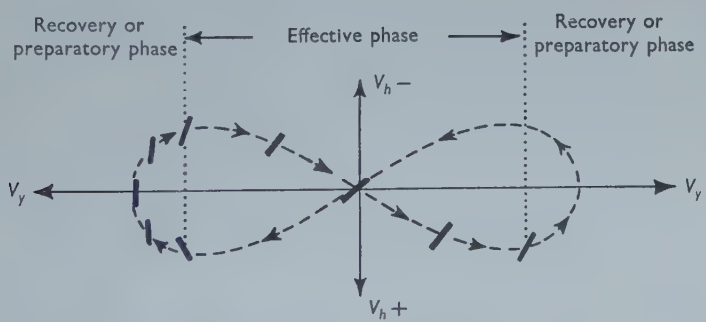
$$dF = C_L[aV_y + bV_h]\, ds, \quad \text{(iii)}$$

$$dT = C_L[cV_y + aV_h]\, ds. \quad \text{(iv)}$$

As shown in Table 3 the propulsive coefficient of transverse displacement a is zero at 0°, rises to a maximum of 0·5 at 45° and then declines to zero again at 90°. The corresponding coefficient for longitudinal displacement b has a minimum value of 1·0 at 0° and rises to a maximum of 2·0 at 90°; a is always substantially less than b.

Table 3

	0°	15°	30°	45°	60°	75°	90°	120°	135°	180°
a	0	0·25	0·43	0·5	0·43	0·25	0	−0·43	−0·5	0
b	1·0	1·07	1·25	1·5	1·75	1·93	2·0	1·75	1·5	1·0
c	2·0	1·94	1·8	1·5	1·25	1·07	1·0	1·25	1·5	2·0



Text-fig. 5. Diagram illustrating the orientation and displacement (relative to the head) of a distal element during the recovery and effective phases of its cycle. In the former V_h is negative, θ is small and V_y is low; during the effective phase V_h is positive, θ is large and V_y is high.

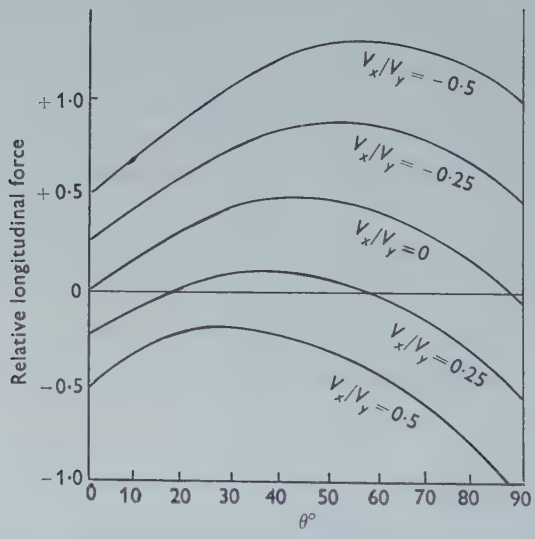
Propulsive forces

When the element is travelling along its figure-of-eight path near its position of maximum transverse displacement it is inclined at a small angle to the axis of propulsion, its transverse velocity is low and it is travelling *forward* relative to the head (i.e. V_h is negative) (see Text-fig. 5). All these characteristics indicate that during these phases of its motion the propulsive force exerted must be negligibly small if not negative. On the other hand, when the element carries out its main transverse sweep its angle of inclination is relatively large, its transverse velocity is high, and it is travelling away from the head (i.e. V_h is positive), Text-fig. 5. All these characteristics indicate that it is during these latter phases that the element exerts its main propulsive effort; the whole cycle can be divided into two effective

phases and two preparatory or recovery phases. During each complete cycle the values of θ, V_h and V_y are varying, but a rough comparison between the two phases can be obtained by substituting in equation (iii) average values derived from observational data.

Effective phase	Preparatory phase
$V_y = 5 \cdot 10^{-2}$ cm./sec.	$V_y = 1 \cdot 10^{-2}$cm./sec.
$V_h = 1 \cdot 10^{-2}$ cm./sec.	$V_h = -1 \cdot 10^{-2}$ cm./sec.
$\theta = 60^\circ$	$\theta = 10^\circ$
$dF = 4 \cdot 10^{-2}\, C_L\, ds$	$dF = -1 \cdot 10^{-2}\, C_L\, ds$

From a similar line of argument, it follows that elements lying towards the front end of the tail can exert little or no propulsive thrust; their angles of inclination and their transverse speeds are low, and they do not exhibit longitudinal movement relative to the head. It may, therefore, be concluded that the propulsive properties of an element of the tail of a bull's spermatozoon increase progressively the nearer the element lies towards the distal end of the tail. The function of the proximal elements is discussed later.



Text-fig. 6. Graph showing the effect of forward displacement and change in angle θ° of inclination on the longitudinal force developed by element. A resultant forward thrust only develops if the speed of forward displacement (V_x) is less than about one-quarter to one-third of its transverse speed.

When a spermatozoon is propelling itself freely through the water, the thrust developed by a distal element during its effective phase will be less than that of a cell whose head is fixed to the slide, whilst the drag during the recovery phase will be increased. Since the two phases are of approximately the same duration and the forces proportional to the element's velocity, the limit of propulsive speed is reached when the average thrust during the effective phase is equal to the average drag during the recovery phase. The effect of superimposing a longitudinal displacement on an element's transverse displacement is shown in Text-fig. 6. If the head of a spermatozoon is moving forward with velocity V_p, whilst an element is

executing its figure-of-eight movement relative to the head, the element's *forward* longitudinal velocity will be V_h+V_p during the recovery phase and V_h-V_p during the effective phase. The resultant propulsive thrust will be zero when

$$aV_y+b(V_h-V_p)=b_1(V_h+V_p).$$

If the average value of $\theta=45°$ during the effective phase and 10° during the recovery phase, whilst $V_h=0{\cdot}25\,V_y$, the resultant thrust will be zero when $V_p=0{\cdot}25V_y$; in other words, an element of this type cannot propel itself at a velocity greater than one-quarter of its transverse speed. The average value of the transverse velocity of a distal element of a bull's spermatozoon is of the order of 500 μ/sec., thus giving a maximum propulsive speed of about 125 μ/sec. As calculated in this way no regard is paid to the fact that the distal elements have to overcome the drag of the proximal elements and of the head. In the case of a sea-urchin, where the duration of the recovery phase is extremely short and V_h is zero, the resultant thrust becomes zero when $V_p=aV_y/b$. If a/b is given its maximum value of 0·33 and V_y is 600/sec., the calculated speed of propulsion (200/sec.) is very close to the observed value (Gray & Hancock, 1955).

Transverse forces

An element of the tail cannot elicit a propulsive thrust from the water without encountering transverse resistance; the development of both forces implies that equal but opposite forces are operating against other parts of the cell. In Text-fig. 7 a distal element (C) is moving towards the right side of the axis of propulsion (xx_1), and eliciting a reaction (R_c) which has a propulsive component (F_c) and a transverse component (T_c); under such conditions the movements of the rest of the cell relative to the water must be such as to produce a drag force equal, and opposite to F, and a transverse force equal, but opposite to T_c. These conditions can be satisfied if there are two other elements, or groups of elements situated anteriorly to C, one of which (A) is moving towards the right side and the other (B), towards the left side of the propulsive axis; the whole length of the tail must therefore form more than one complete 'wave-length'; at some phases of the cycle there may be four centres of transverse pressure instead of three (see Text-fig. 8).

As already indicated, the transverse force exerted by an element depends on its speed of transverse and longitudinal displacement and on its angle of inclination. The quantitative relationship between these three factors is shown in Text-fig. 9. High transverse forces are elicited by elements with low angles of inclination and by movement of the element forwards along the axis of propulsion; these conditions exist in the proximal regions of the tail; on the other hand, relatively low transverse forces are developed by the distal elements where the angle of inclination is relatively high. The main dynamic function of the elements lying towards the anterior end of the tail (together with the head and middle piece) is to provide a stable fulcrum against which the distal elements can exert their propulsive effort. As a propulsive system the spermatozoon of a bull is comparable with a fish, such as a trout, where the front end of the body provides the fulcrum against which the tail

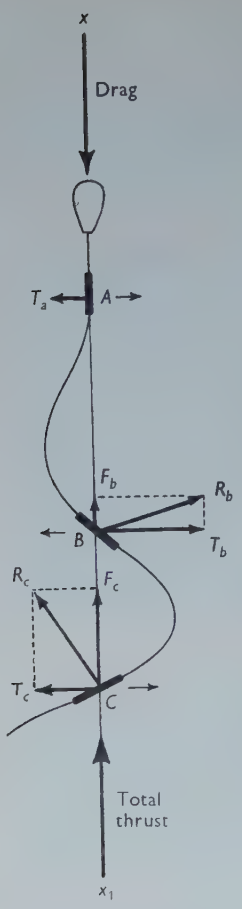


Fig. 7

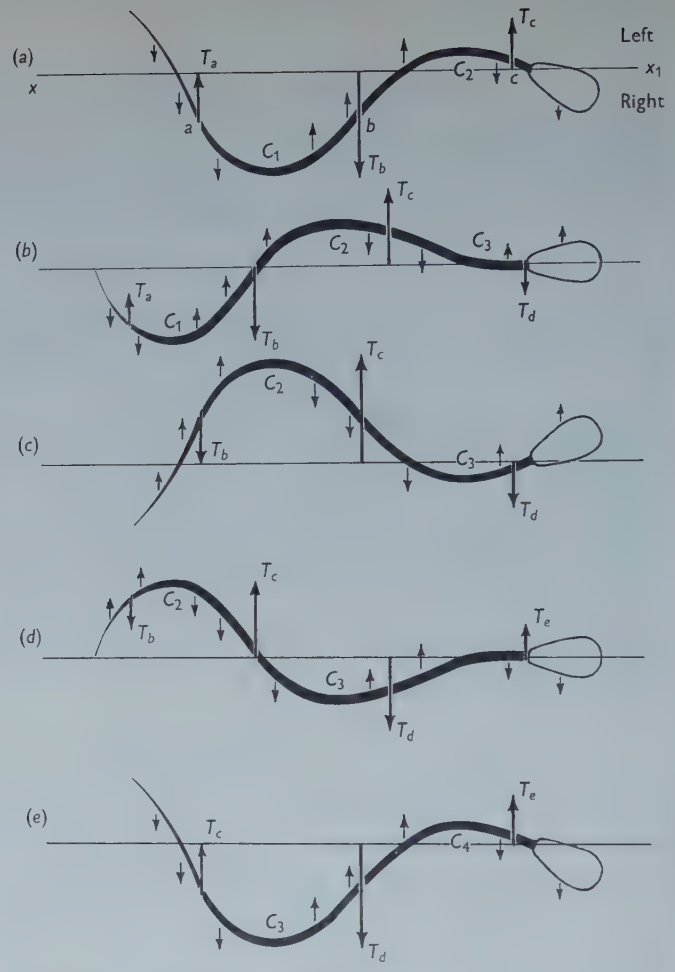


Fig. 8

Text-fig. 7. Diagrammatic representation of the forces exerted by the water against three groups of elements of the tail. If a distal group of elements (C) is moving to the right and eliciting a reaction R_c, the motion of the rest of the spermatozoon relative to the water must be such as to elicit a reaction equal but opposite to R_c. This condition is satisfied if (as in the figure) there is an anterior group of elements (A) (together with the head) moving to the right and eliciting a reaction T_a, and an intermediate group of elements (B) moving to the left and eliciting a reaction R_b. The resultant of the forward components (F_b and F_c) of R_b and R_c is equal to the total backward drag of the whole cell, whilst the resultant of the transverse forces (T_a, T_b, T_c) is zero. The small arrows show the direction of lateral movement of the three groups of elements.

Text-fig. 8. Diagram showing the general distribution of transverse forces as a bending wave passes along the tail. Regions of maximum curvature are shown at C_1, C_2, C_3 and C_4. The directions of the movement of the various regions of the tail are shown by the small arrows.

(8*a*). All elements posterior to C_1 are moving towards the right side of the axis (xx_1) of progression; elements between C_1 and C_2 are moving to the left, whilst the head and elements anterior to C_2 are moving to the right. The centres of transverse pressure against these three regions are at *a*, *b* and *c*, respectively, and the forces acting are T_a, T_b and T_c, their resultant being zero.

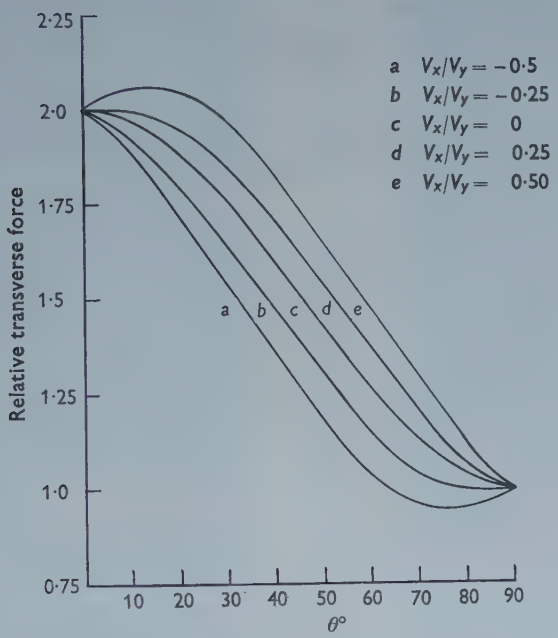
(8*b*). The phase of movement has advanced by one-quarter cycle, the regions of maximum contraction (C_1 and C_2) lie nearer to the tip of the tail. A new region of maximum contraction is developing at C_3, and all elements anterior to C_3 are moving to the left of the axis and elicit the reaction T_d. The resultant of T_a, T_b, T_c and T_d is zero.

(8*c*). One-quarter cycle ahead of Text-fig. 8*b*. The form of the tail and the distribution of transverse forces are the mirror images of those in Fig. 8*a*.

(8*d*). One-quarter cycle ahead of Text-fig. 8*c*: the figure is the mirror image of Text-fig. 8*b*.

(8*e*). The tail has completed one whole cycle.

and caudal fin exert their propulsive effort; the spermatozoon of a sea-urchin is equivalent to an eel or snake where the motion relative to the water, is more nearly the same for all elements. In all cases the form of the movements is probably closely related to the length and inherent flexibility of the body.



Text-fig. 9. Graph showing the effect of longitudinal displacement on the total transverse force elicited by an element. This force increases as the rate of *forward* movement increases. At low angles of inclination the relative value of the transverse force approximates to twice that characteristic of high angles.

SUMMARY

1. The maximum extent to which an element of the tail of a bull's spermatozoon bends during its contractile cycle is not the same for all elements; the nearer the element lies towards the tip of the tail the greater is the amount of bending.

2. The phase difference between successive elements varies along the length of the tail; and consequently the speed of propagation of the bending wave decreases as the latter moves backwards.

3. The amplitude of transverse movement relative to the head increases progressively along the tail towards the distal end.

4. Distal elements execute figure-of-eight movements relative to the head.

5. The frequency of the bending cycles and the propulsive velocity of the whole cell vary considerably. The average frequency for thirty-one cells was 9·1/sec., and the average propulsive speed for 235 cells was 94 μsec.

6. Cells moving freely in water 'flashed' with a frequency similar to that of the bending waves. The rotation of the head about its longitudinal axis appears to be

due to the fact that all elements of the tail are not executing their transverse movements in exactly the same plane during the whole of their contractile cycles.

7. The rate at which an element can propel itself forward cannot be greater than about one-third to one-quarter of its average transverse velocity.

8. The distal elements of the tail exert their propulsive effort against the fulcrum provided by the proximal elements.

9. It is impracticable to relate the speed of propulsion to the form and speed of propagation of the waves passing along the tail.

The photographs for this paper were taken by Mr K. C. Williamson to whom the author wishes to express his very sincere thanks.

REFERENCES

BROWN, R. H. J. & POPPLE, J. A. (1955). Multiple flash photography. *Med. Biol. Ill.* **5**, 23–28.
GRAY, J. (1955). The movement of sea-urchin spermatozoa. *J. Exp. Biol.* **32**, 775–801.
GRAY, J. & HANCOCK, G. J. H. (1955). The propulsion of sea-urchin spermatozoa. *J. Exp. Biol.* **32**, 802–14.
HANCOCK, G. J. H. (1953). The self-propulsion of microscopic organisms through liquids. *Proc. Roy. Soc.* A, **217**, 96–121.
ROTHSCHILD, Lord (1953*a*). A new method of measuring the activity of spermatozoa. *J. Exp. Biol.* **30**, 178–99.
ROTHSCHILD, Lord (1953*b*). *Mammalian Germ Cells.* London. 122–133.

EXPLANATION OF PLATES 2–4

PLATE 2

Dark-ground photographs taken on stationary film. Interval between flashes $\frac{1}{80}$ sec. Note transverse displacement of middle piece in photograph 6; transverse movement of head and middle piece in photographs 8–14.

PLATE 3

Dark-ground photographs taken on moving film. Interval between exposures $\frac{1}{66}$ sec. Note increase in maximum curvature and change of shape as a wave (●, +) passes posteriorly along the tail (photographs 2–10). Also note asymmetry of bending on two sides of tail (photographs 6, 10). [In order to reproduce the original photographs it proved necessary to retouch the distal end of the tail].

PLATE 4

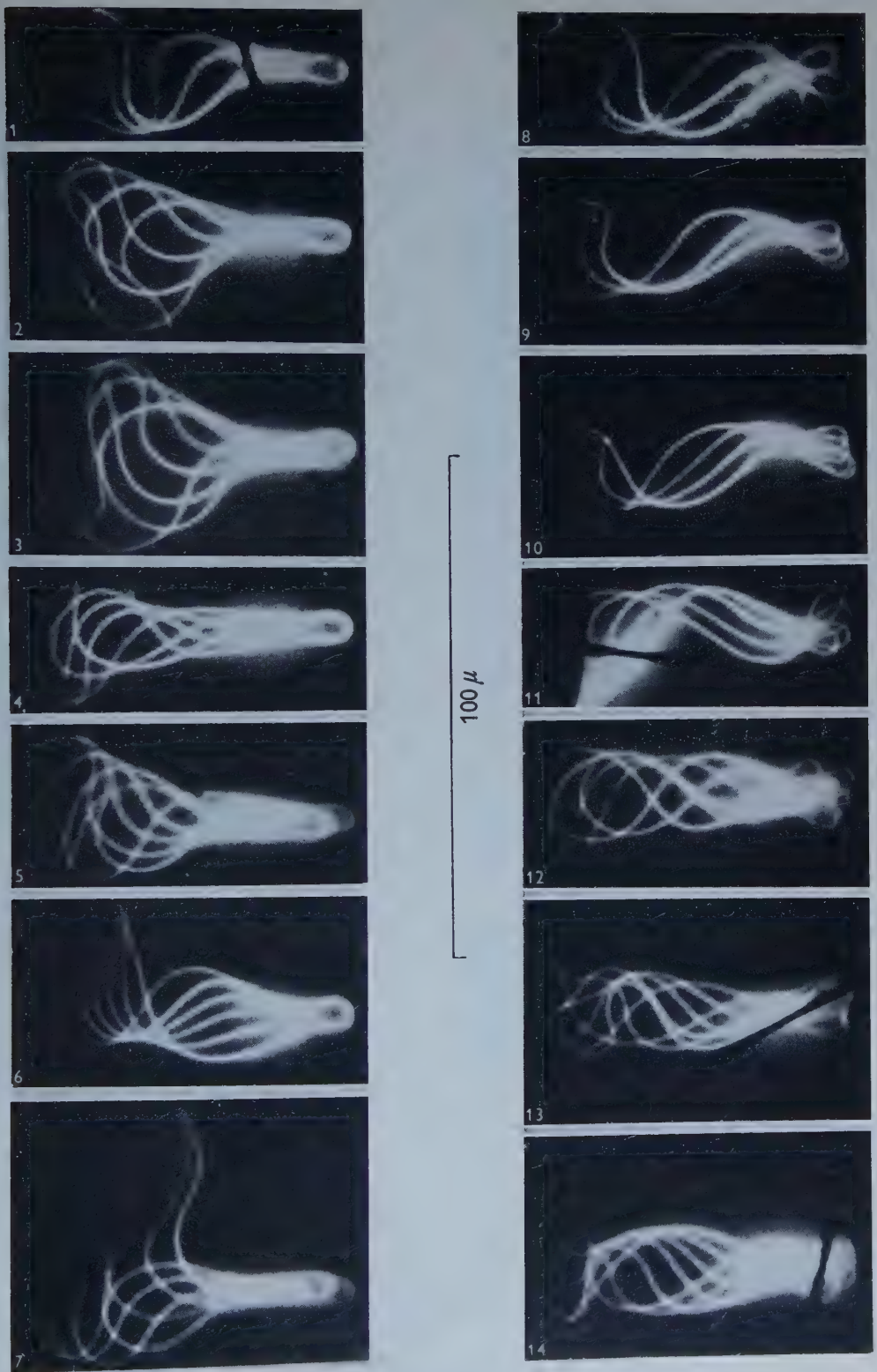
(*a*) Successive dark-ground photographs of the optical envelope of a flashing cell. Exposure $\frac{1}{24}$ sec., interval between exposures $\frac{1}{18}$ sec.

(*b*) Successive dark-ground photographs (exposure $\frac{1}{240}$ sec., interval $\frac{1}{20}$ sec.) of a headless cell when rolling about its main longitudinal axis.

(*c*) Successive photographs (phase contrast—exposure $\frac{1}{288}$ sec., interval $\frac{1}{3}$ sec.) of a cell which 'flashed' each time a region of maximum curvature passed over the distal region of the tail.

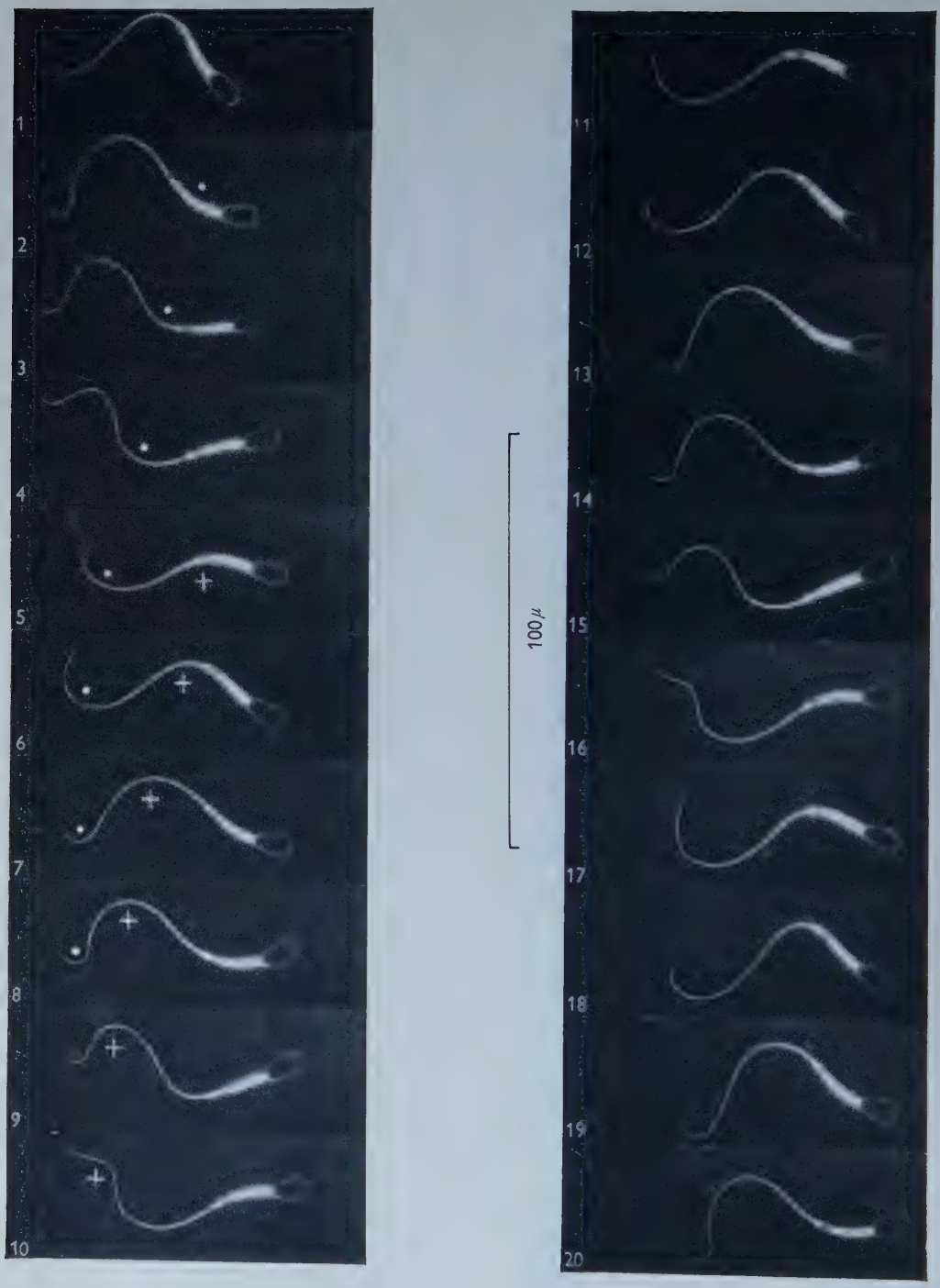
(*d*) Dark-ground photograph (three electronic flashes) of a 'flashing' cell: note that the plane of beat of the distal end of the tail is not the same as that of the rest of the tail.

(*e*) Photograph of four cells exhibiting co-ordinated movement.



GRAY—THE MOVEMENT OF THE SPERMATOZOA OF THE BULL

(*Facing p.* 108)



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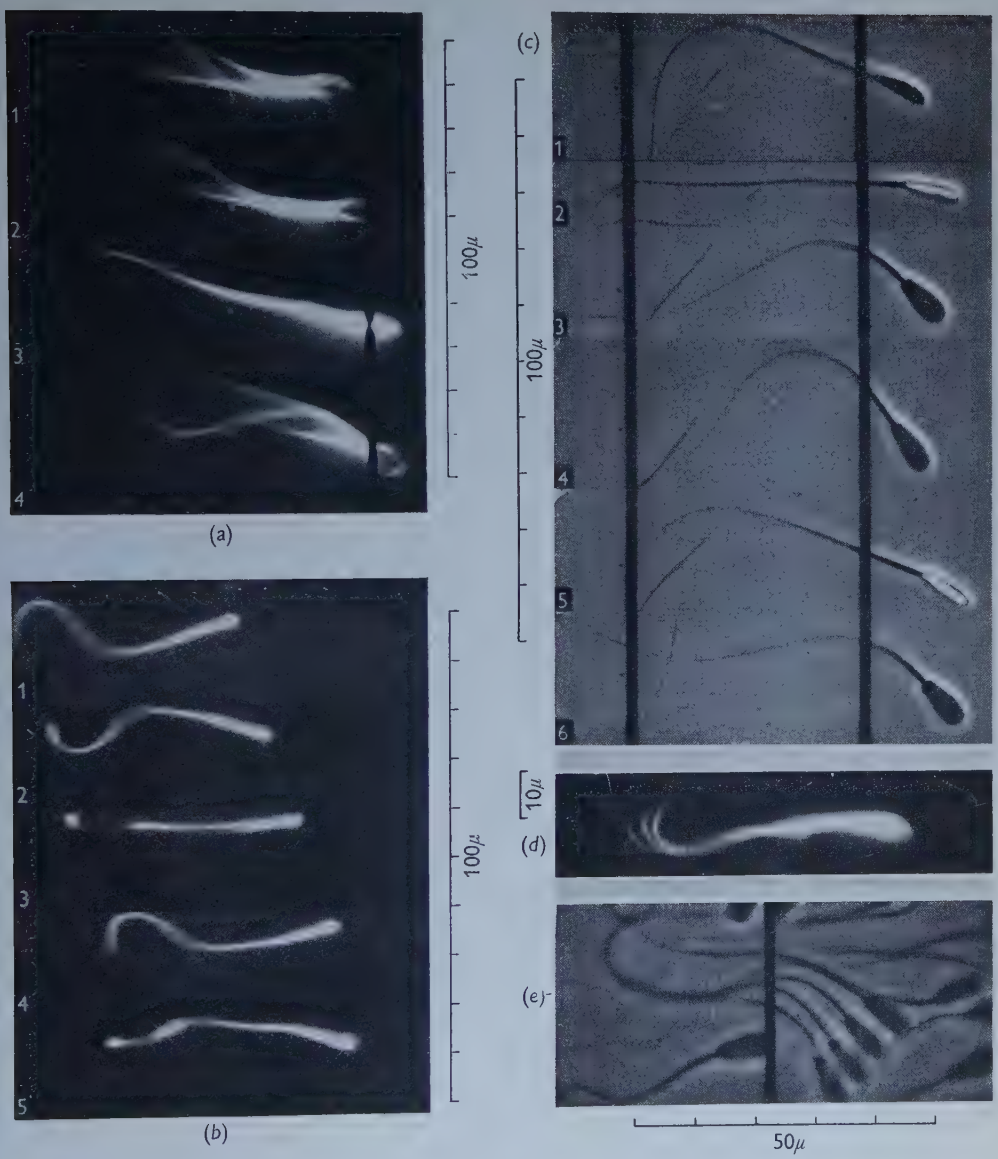

GRAY—THE MOVEMENT OF THE SPERMATOZOA OF THE BULL

THE SPEED OF SWIMMING OF FISH AS RELATED TO SIZE AND TO THE FREQUENCY AND AMPLITUDE OF THE TAIL BEAT

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INTRODUCTION

A fair number of values for the speed of swimming of a representative selection of fish species now exists in the literature. Many of the figures are only estimates; some are accurate measurements, but unfortunately even amongst these the absence of the all-important values of the weight or the length of the specimens concerned renders many of them of little use.

Stringham (1924) reviews and gives references to early work and Gray (1953) summarizes some later results and also gives figures of his own. The earliest recorded experiments seem to be those of Regnard (1893) who allowed small fish to swim in an annular rotating vessel, the speed of which could be controlled by a variable resistance and an electric motor. He rotated the vessel until the swimming fish could only just keep station and then recorded this as the maximum swimming speed. He makes no reference in his account to corrections for a lag between the speed of rotation of the water and that of the container, and in this respect his results may perhaps be suspect. Figures for by far the greatest selection of species are to be found in Magnan (1930). Besides dividing 172 species up into eight great groups according to the relationship between their speed of swimming and the square root of their length, he gives a table of values for eighteen species, recording amongst other things the length of the specimens used and their maximum speed of swimming. The methods he used comprise (i) timing the animal between two fixed points a known distance apart, (ii) using a special camera to make ciné films of the animal swimming and calculating values from these, and (iii) attaching the animal to a speedometer by means of a thread, the speed at which this unrolled giving the fish's speed. He does not say which of these methods is used in any particular instance, or upon what distance of swimming any measurement is based, but regards all the figures as equally reliable. Denil (1937), in one of a series of papers, which deal exhaustively with the problem of constructing fish ladders for surmounting river obstacles, gives some measurements of the speed of various specimens swimming up passes of different design, and calculates from these figures their potential speed in open water. He also calculates the speed at which leaping fish must be moving when they leave the water. Lane (1941), in an entertaining popular article, refers to a device, made by H. E. Thompson and similar to that used in the last of

Magnan's methods, for timing the speed of running out of a line when a fish has been hooked. He gives one value obtained with this for a tuna.

More recently Fry & Hart (1948) have used an apparatus similar to Regnard's in a study of the relationship between speed and water temperature in the goldfish. They make careful allowance for the lag between water and container and give what may be considered definitive figures for a 'cruising speed' that can be sustained for, say, 15–20 min. Davidson (1949) gives figures for salmon maintaining station in a circular rearing tank in which the water was rotating, and Wales (1950) records what is probably the maximum speed of *Catostomus occidentalis*, the western sucker, swimming in a culvert containing rapidly flowing water. Radcliffe (1950) gives more figures for goldfish measured in Fry & Hart's apparatus and also records the effect of clipping off various fins. This he finds slightly increases the maximum speed, in direct contradiction to Regnard's (1893) observation that it materially decreases the speed. Gero (1952) refers to an instrument called the 'Piscatometer'. This allows both the thrust and velocity of a fish hooked during angling to be measured at sea in an open boat. The velocity is measured on a tachometer actuated as the line runs out. The tachometer dial is photographed during this process. He gives figures for two shark and a barracuda.

The results obtained by these various authors are summarized in Table 1, where all the speeds have been expressed in terms of the number of body lengths moved in 1 sec. In those cases where only the weight of the specimen was given, weight has been converted into length using the relationships given in Hecht (1916). As can be seen, the maximum speed actually recorded hitherto is that for the tuna, which Lane gives as travelling at 13·4 times its own length per second. The highest figure of all is that of Denil for a trout moving at 19·4 times its own length per second when leaving the water; but this is derived by calculation from a report of a trout of 25 cm. having leaped to a height of 1 m.

Without of course performing a great many measurements it is almost impossible to tell whether a particular fish is swimming at anything like its maximum speed. If, however, the one high figure given by Denil is taken as representing a rapid dart sustained only for a second or two, then it is seen that amongst the remainder there is a group of figures in the neighbourhood of 10 lengths per second

Bleak	10·0	Circular tank	Pike	12·7	Photography
Trout	10·0	In fish pass	Carp	12·6	
Tuna	13·4	Line running out	Dace	9·2	
Sucker	9·8	Culvert			
Barracuda	9·4	'Piscatometer'			

These have been measured by a diversity of means, and the first five at least are based on a sustained period of swimming. It seems reasonable to assume that this speed of 10 lengths per second is the maximum that can be sustained for a period of about 1 min. The remaining figures in the table probably represent speeds sustainable for longer than this interval, or are based upon fish not exerting their maximum effort.

Table 1 *Published speeds of various fish*

Authority	Common name	Species	Speed in lengths/sec.	Data given	Remarks
Regnard, 1893	Carp	*Cyprinus carpio*	5·2	6 g., fish at 59 cm./sec. 5 g., fish at 52 cm./sec. 5 g., fish at 22 cm./sec.	(Carpe)
	Bleak	*Alburnus alburnus*	10·0	1 g., fish at 50 cm./sec.	(Ablette)
	Chub	*Squalius cephalus*	2·0	15 g., fish at 24 cm./sec.	(Chevaine)
Magnan, 1930	Sea trout	*Salmo trutta*	2·7	34·1 g., fish at 92 cm./sec.	(Truite de mer)
	Mackerel	*Scomber scombrus*	3·2	25·2 g., fish at 81 cm./sec.	(Maqereau)
	Twaite shad	*Alosa finita*	2·5	29·7 g., fish at 75 cm./sec.	(Alose fint)
	Perch	*Perca perca*	3·6	18·4 g., fish at 66 cm./sec.	(Perche)
	Meagre	*Sciaena aquila*	3·8	29·5 g., fish at 113 cm./sec.	(Maigre)
	Whiting	*Gadus merlangus*	1·3	17·7 g., fish at 23 cm./sec.	(Whiting)
	Bib or Pout	*G. luscus*	3·3	16·5 g., fish at 55 cm./sec.	(Tacaud)
	Grey mullet	*Mugil capito*	2·3	26·0 g., fish at 61 cm./sec.	(Mulet céphale)
	Rudd	*Scardinius erythrophthalmus*	6·0	18·8 g., fish at 114 cm./sec.	(Rotengle)
	Lesser weever	*Trachinus vipera*	1·8	22·4 g., fish at 40 cm./sec.	(Vive)
	Hake	*Merluccius vulgaris*	3·5	23·7 g., fish at 79 cm./sec.	(Merlus)
	Pike	*Esox lucius*	3·9	37·8 cm., fish at 148 cm./sec.	(Brocket)
	Red gurnard	*Trigla pini*	2·4	19·2 cm., fish at 47 cm./sec.	(Rouget)
	John Dory	*Zeus faber*	1·6	19·7 cm., fish at 30 cm./sec.	(Saint Pierre)
	Black bass	*Micropterus salmoides*	4·1	21·3 cm., fish at 88 cm./sec.	—
	Gurnard	*Trigla* sp.	5·0	26·2 cm., fish at 131 cm./sec.	(Grondin corbeau)
	Norway haddock	*Sebastes dactylopterus*	3·6	26·8 cm., fish at 98 cm./sec.	(Sébaste)
Denil, 1937	Trout	*Salmo fario*	10·0	35 cm., fish at 3·5 m./sec.	—
	Salmon	*S. salar*	6·4	75 cm., fish at 4·79 m./sec.	—
	Salmon	*S. salar*	5·8	85 cm., fish at 4·95 m./sec.	—
	Salmon	*S. salar*	8·4	80 cm., fish leaping 2 m.	Calculated from reported data
	Trout	*S. fario*	19·4	25 cm., fish leaping 1 m.	
	Salmon	*S. salar*	8·0	*ca.* 75 cm., fish at 6 m./sec.	—
Lane, 1941	Tuna	*Thunnus thynnus*	13·4	60 lb. fish travelling at 44 m.p.h.	—
Fry & Hart, 1948	Goldfish	*Carassius auratus*	6·36	Fish of average wt. 4·37 gm. swimming at 100 ft./min.	Average of many measurements and sustained for 20–25 min. at 25° C.

Table 1 (*continued*)

Authority	Common name	Species	Speed in lengths/sec	Data given	Remarks
Davidson, 1949	Salmon	*Salmo salar*	4·0	5·2 cm. fish, av. speed 21·6 cm./sec. 4·3 cm. fish, av. speed 16·7 cm./sec. 3·2 cm. fish, av. speed 13·1 cm./sec.	Each an average of twenty fish
Wales, 1950	Western sucker	*Catostomus occidentalis*	9·8	Fish 12–14 in. swimming at 2 ft./sec. in water flowing at 8·6 ft./sec.	Sustained 5 sec.
Radcliffe, 1950	Goldfish	*Carassius auratus*	3·4	3·38 body lengths/sec.	Normal
	Goldfish	*C. auratus*	4·9	4·90 body lengths/sec.	After clipping fins
Gero, 1952	Southern ground shark	*Carcharinus leucas* (=*commersonii*)	3·4	60 in., fish at 17·1 ft./sec.	'Piscatometer'
	Southern ground shark	*C. leucas* (=*commersonii*)	3·9	21 lb., fish at 13·3 ft./sec.	
	Spotted jewfish	*Promicrops itaiara*	1·8	17 lb., fish at 5·7 ft./sec.	
	Lemon shark	*Negaprion brevirostris*	1·3	72·5 in., fish at 8·0 ft./sec.	
	Barracuda	*Sphyraena barracuda*	9·4	51·0 in., fish at 40·0 ft./sec.	
Gray, 1953	Rainbow trout	*Salmo irideus*	8·5	20 cm., fiih at 1·7 m./sec.	Photography of darting fish
	Pike	*Esox lucius*	12·7	16·5 cm., fish at 2·1 m./sec.	
	Pike	*E. lucius*	7·5	20 cm., fish at 1·5 m./sec.	
	Carp	*Cyprinus carpio*	12·6	13·5 cm., fish at 1·7 m./sec.	
	Rudd	*Scardinius erythrophthalmus*	5·9	22 cm., fish at 1·3 m./sec.	
	Dace	*Leuciscus leuciscus*	9·2	18·15 cm., fish at 1·7 m./sec.	
	Salmon	*Salmo salar*	9·2	3 ft., fish assumed jumping 6 ft.	—
	Salmon	*S. salar*	12·2	3 ft., fish assumed jumping 10 ft.	—

Some indication of the rate during a very prolonged period of swimming may be obtained from marking experiments on migrating fish. For instance Dahl & Sømme (1936) describe how a fish marked at Titran in Norway was recovered at Drammenfiord, 1100 km. away, after 11 days. The specimen was 85 cm. long and this represents an average rate of progression of 1·4 lengths per second. As the route would almost certainly not be straight, and the migration did not necessarily start and finish at the times of release and capture, it is probable that this figure is low.

EXPERIMENTAL METHOD

It is reasonable to suppose that the speed at which a particular specimen travels depends upon several properties—the form of the body, its surface texture, the size of the specimen and the frequency and amplitude of beating of the tail (or of undulating the body or of moving the paired fins). The form of the body is known for all the species listed, as is the approximate length. Hitherto, however, no record has been made of the relationship between the speed and the frequency or the amplitude of tail beat or body movement. If speed could be related to these variables and limits could be ascribed to them, then it should be possible to forecast with accuracy the speed attainable by any particular specimen.

With this end in view it seemed desirable to make, if possible, more accurate measurements of the speed of swimming of various fish, and to relate this clearly to at least the three factors size, frequency and amplitude. An apparatus was therefore devised in which the fish was harnessed to the end of a light arm free to rotate in the horizontal plane. The fish was allowed to swim in a circular tank of water, and the rotation of the arm was recorded electrically on a smoked drum. The fish's speed was calculated from this recorded speed of rotation and the length of the arm. Attempts were made to obtain a photographic record of the fish's body movements over an arc of the circle in order to correlate the frequency of tail beat with the speed. While the preliminary results obtained with this apparatus were of some interest, it became clear that movement of the fish was made unnatural by its having to swim in a curved path, and to some extent the arm which had to be carried round constituted a load on the fish. It was consequently decided to develop an apparatus in which a free-swimming fish could be kept under continuous observation, while at the same time being able to travel unlimited distances.

The principle adopted was that described by Hardy & Bainbridge (1954) and utilized to facilitate a study of the vertical migrations of plankton organisms. Essentially it consists in moving the animal's environment bodily in a direction opposite to, and at the speed of, any movement that the animal cares to make. The 'fish wheel', as the apparatus built became known, consisted in the first instance, as shown in Fig. 1, of a horizontal circular tube of rectangular cross-section, built up of pieces of $\frac{1}{8}$ in. transparent Perspex. The tube so formed was $2\frac{1}{2}$ by 3 in. in cross-section and the internal diameter of the wheel was 2 ft. It was supported on three $\frac{1}{2}$ by $3\frac{1}{2}$ in. diameter ball races set on edge under the centre of the tube, each with a rubber tyre, and was secured to a central bearing, about which it could rotate,

by three wooden spokes and a boss. To facilitate cleaning, the entire top of the tube could be unbolted and removed, but one sector, 6 in. long, was secured separately to provide an opening for purposes of filling the tube and introducing the fish. Relative movement between the tube and its contained water was prevented by two Perspex sliding doors, moving vertically and fitting closely into guides on each side of the tube. These were made to open and close by means of the cam and rod mechanism shown in Fig. 2. The cam was arranged to keep a door fully open over

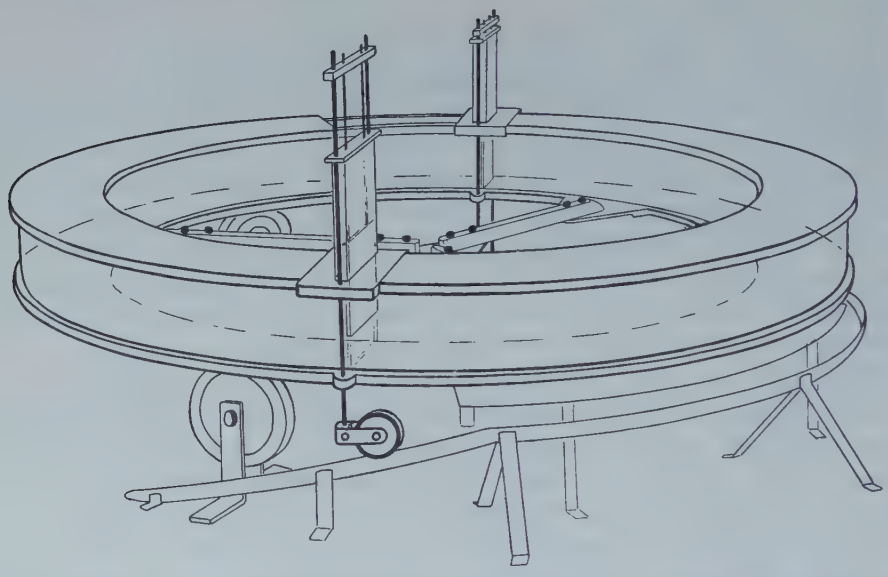

Fig. 1. Perspective view of small fish wheel. The animal swims on the extreme right where the vertical doors pass in the open position. On the left the doors close, thus locking the water to the wheel.

an arc of 90° and the opening and closing movements each occupied about 45°. The doors being diametrically opposite each other this arrangement ensured that one was always closed, while the other was open to allow for the passage of the fish. Radial black lines 10 cm. apart were drawn on the underside of the floor of the tube and showed distinctly against a white background. They were used to simplify analysis of the film records taken. A simple speedometer driven by one of the rotating ballraces was also arranged to be in the field of the camera. It was roughly calibrated by rotating the whole apparatus at various known speeds. This apparatus was used to obtain about half the records described here. The remainder, all of larger specimens, were obtained with the larger electrically operated apparatus described in the succeeding paper (Bainbridge & Brown, 1958).

For both types of apparatus records were taken with a ciné camera mounted directly above the point at which the fish swims. Illumination was by two or three Mazda no. 1 Photofloods about 24 in. from the fish, and the field of view included the fish, the speed indicator and a time marker. Generally, it was found that twenty-

four or forty-eight frames per second on 35 mm. film was sufficient to permit analysis of tail beat, etc., but occasionally 16 mm. films were taken at speeds up to sixty-four frames per second.

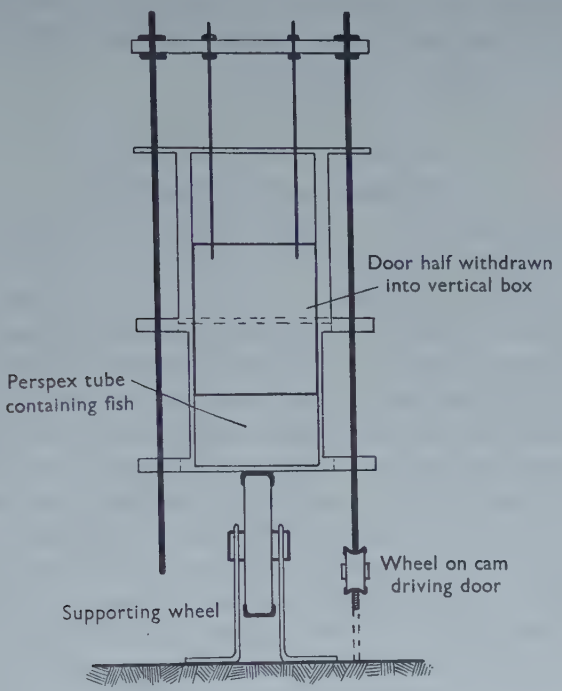

Fig. 2. Half-section of the wheel with a vertical door half-open.

In a typical experiment the tube is completely filled with water at room temperature, great care being taken to exclude bubbles. The fish is introduced and the removable cover bolted on. After the fish has been allowed to settle down the apparatus is rotated until he is over the white background where the doors pass by in the open position. As soon as he starts to swim the wheel is now rotated at the same speed, but in a direction opposite to his movement, thus keeping him stationary relative to the observer. As soon as the speedometer indicates that he is swimming steadily at a speed that it is desirable to record, the ciné camera is started and a length of film lasting some 3 or 4 sec. is taken. This procedure is repeated at intervals whenever the fish obliges by swimming at a required speed. In this way a permanent record of his speed and body movement is made for subsequent analysis without any extravagant expenditure of film. If the fish declines to swim it is sometimes possible to stimulate him to do so by moving a background of vertical stripes close to the side of the tube. Often the highest speeds are obtained immediately after he has been put into the apparatus, but they may be induced by flashing lights, waving a hand or tapping on the wall of the tube.

The lengths of film so obtained are analysed by projecting frame by frame on to a paper and plotting successive positions of the fish's tail in relation to the transverse black lines on the floor of the tube. Knowing the number of frames run per second it is possible to obtain directly from such a plot (i) the exact speed of swimming through still water over any given period, (ii) the frequency, and (iii) the amplitude of beating of the tail. The length of the specimen is taken at the time of the experiment, but can also be measured from the film.

EXPERIMENTAL RESULTS

The dace, Leuciscus leuciscus

The results obtained using dace of different lengths are first examined. Fig. 3A shows the relationship between speed and frequency of tail-beat of a fish 17·5 cm. long from the tip of the snout to the most posterior part of the tail fin. Each of the thirty-two points was obtained by analysing a length of film taken when the specimen was swimming steadily at the speed indicated. Each value derives from at least 50 cm. of swimming and generally from much more. It is clear from this graph that the relationship between speed and frequency could be represented by a straight line, which would not, however, pass through the origin. The graph for a 24·0 cm. specimen (Fig. 3B) confirms this, and also shows that the larger fish travels faster at any given frequency. This same trend is shown in the opposite direction in Fig. 3C and D, representing the results for 9·0 and 5·2 cm. specimens, respectively. These two graphs further make it clear that the speed/frequency relationship is in fact linear only down to about 5 beats per second, below which it departs from linearity and presumably passes through the origin.

The direct dependence of speed upon the length of the specimen is revealed if the measured speeds are divided by the size of the fish and thus expressed as body lengths per second. These values, derived for all specimens, are plotted against frequency in Fig. 3E, which shows the observations not only for the 24·0, 17·5, 9·0 and 5·2 cm. specimens, but also those for three other fish measuring 8·5, 6·6 and 3·6 cm. In order to avoid confusion in the graph reproduced, the various sizes of fish have not been given distinguishing symbols, but when this is done and a line is drawn through the points, it can be seen (i) that for each size of fish the points are evenly distributed on either side of the line, and (ii) that it is the smaller specimens that reach the higher relative speeds and higher frequencies of tail beat, the maximum being 20 body lengths per second at 25 beats per second.

The exact form of the line describing the speed/frequency relationship is made clearer by examining the relation between frequency and distance travelled per beat. This is shown non-dimensionally in Fig. 4 for all the dace measured. The distance travelled per beat is constant above a frequency of 5 or 6 beats per second but below this value it diminishes. It follows that the speed/frequency relationship in Fig. 3E must be linear from a frequency of 5 or 6 upwards but must be curved at lower frequencies. The three very high points between frequencies 1 and 2 (Fig. 4) may be due to gliding between beats. At these low frequencies beats are often separated

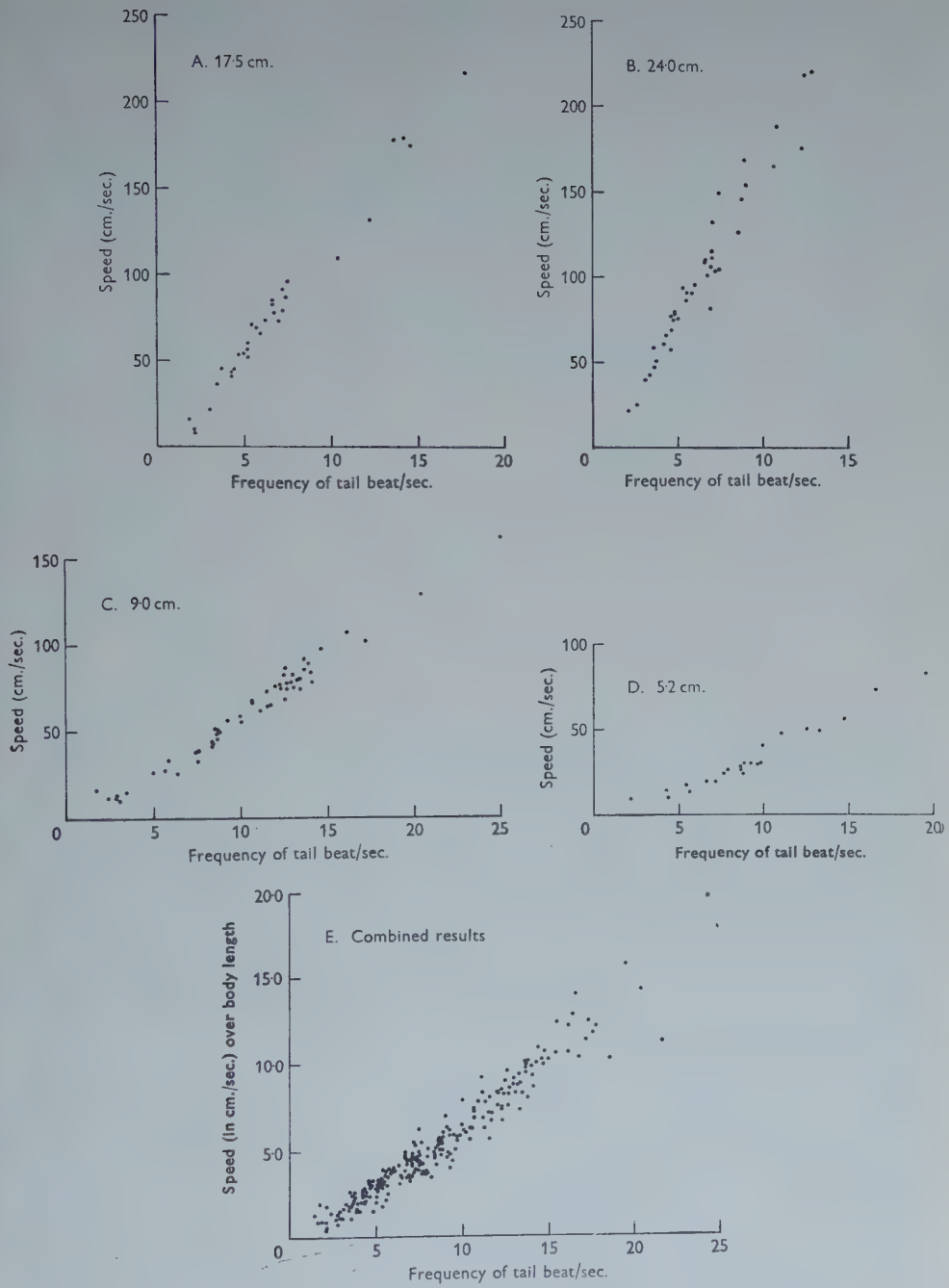

Fig. 3. Relationship between speed of swimming and frequency of beating of the tail for specimens of the dace (*Leuciscus leuciscus*). A 17·5, B 24·0, C 9·0, D 5·2 cm. long. E shows these results (and further information for specimens 8·5, 6·6 and 3·6 cm. long) with the speed expressed non-dimensionally as body lengths per second.

by periods when the fish glides without moving the tail; this possibly allows a maximum distance to be obtained from each beat but only, of course, a very low speed.

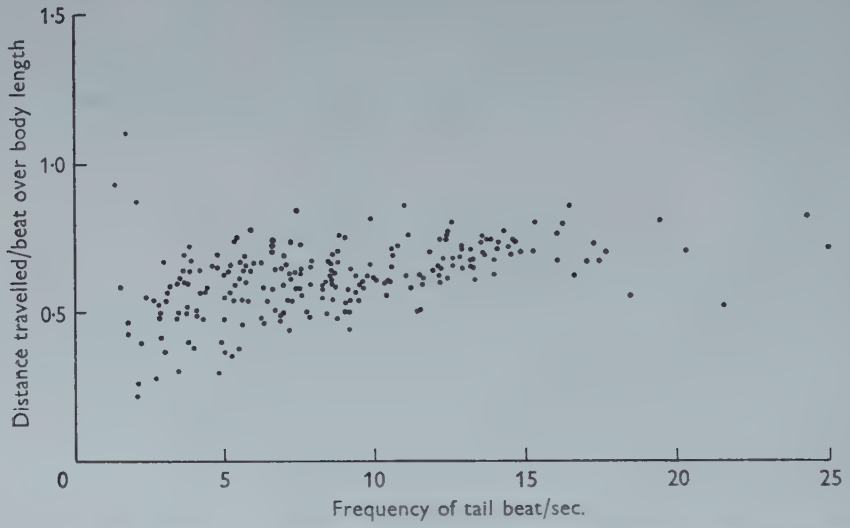

Fig. 4. Relationship between the distance travelled per beat divided by body length and frequency of beating of the tail for the 7 specimens of the dace shown in Fig. 3E.

There is a good deal of scatter amongst the points in both Figs. 3 and 4. Some of this may result from inaccuracies inherent in the experimental method: either a variable leakage of water past the doors, variations in the camera speed or errors in measuring the distances swum. Some may also be due to the inertia of the swimming fish. If the specimen was accelerating slightly some of the propulsive energy would be used in overcoming inertia and the speed measured would be less than when a steady speed was reached at that particular frequency of beating. Similarly, if the fish was decelerating then the speed measured at a particular frequency would be greater than the steady speed. While every care was taken to make records only at steady speeds, slight variations might perhaps escape detection. Most of the scatter can, however, be shown to be dependent upon variations in amplitude of the tail beat.

It did not prove possible to measure amplitude with any accuracy in the smaller specimens, but in the larger ones this was relatively easy. The average was made of ten randomly selected measurements taken from the plotted course of the fish's tail for each length of film representing a period of steady swimming. The distance measured was from one extreme lateral position of the tail to the line joining the preceding and succeeding extreme positions on the opposite side of the body. This gives the tip-to-tip amplitude which, while varying somewhat during any recorded period of steady swimming, was generally fairly constant. Fig. 5 shows the distance travelled per beat plotted against the amplitude for the 24·0, 17·5 and 9·0 cm. specimens. As would be expected, the distance travelled per beat increases with

increase in amplitude, and the relationship appears to be linear between the amplitude limits of 1 and 6 cm.

These limits are interesting. In no case does the amplitude exceed one-quarter of the body length. Amplitudes normally range between 4·5 and 5·5 cm. for the 24·0 cm. specimen, between 3·0 and 4·0 cm. for the 17·5 cm. specimen, and between 1·0 and 2·0 cm. for the 9·0 cm. one. At frequencies above 5 beats per second the ratio of mean amplitude to body length is about 1:5 (Table 2, p. 123) and the ratio of maximum amplitude to body length is about 2:9.

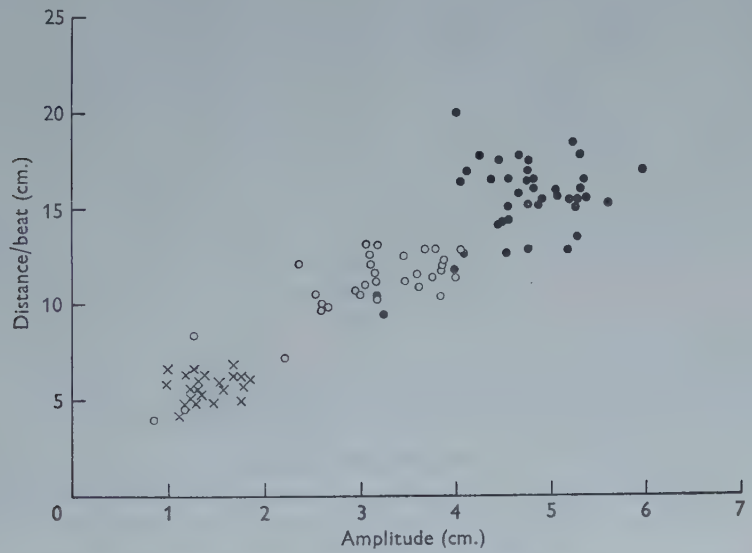

Fig. 5. Relationship between distance travelled per beat and amplitude for 3 dace measuring 24·0 cm. (black circles), 17·5 cm. (white circles) and 9·0 cm. (crosses).

As shown in Fig. 6, for the 24·0 and 17·5 cm. specimens only, amplitude increases rapidly with frequency from the lowest values recorded up to about 5 beats per second, but after this remains steady, with possibly a slight gradual decline at higher frequencies. In this may lie the explanation of the form of the speed/frequency graph shown in Fig. 3E. At frequencies below 5 beats per second the distance travelled per beat will be low by reason of the reduced amplitude; but the amplitude will increase with increasing frequency until at about 5 beats per second the amplitude reaches its maximum value and the distance travelled per beat becomes constant; thereafter the speed/frequency relationship will be linear.

If an equal degree of variability of amplitude is characteristic of all frequencies and if speed is related to amplitude multiplied by frequency, then the variability of speed at high frequencies might be expected to be greater than that at low ones. This may account for the slightly fan-like distribution of points in the speed/frequency graphs.

The final factor limiting the maximum speed attainable by any particular specimen would thus appear to be the frequency of beating of the tail. Fig. 7 shows the

maximum frequencies recorded for the various specimens studied (black circles). While there is no means of knowing that these are really the absolute maximum frequencies that may be reached by these animals there seems little reason to doubt

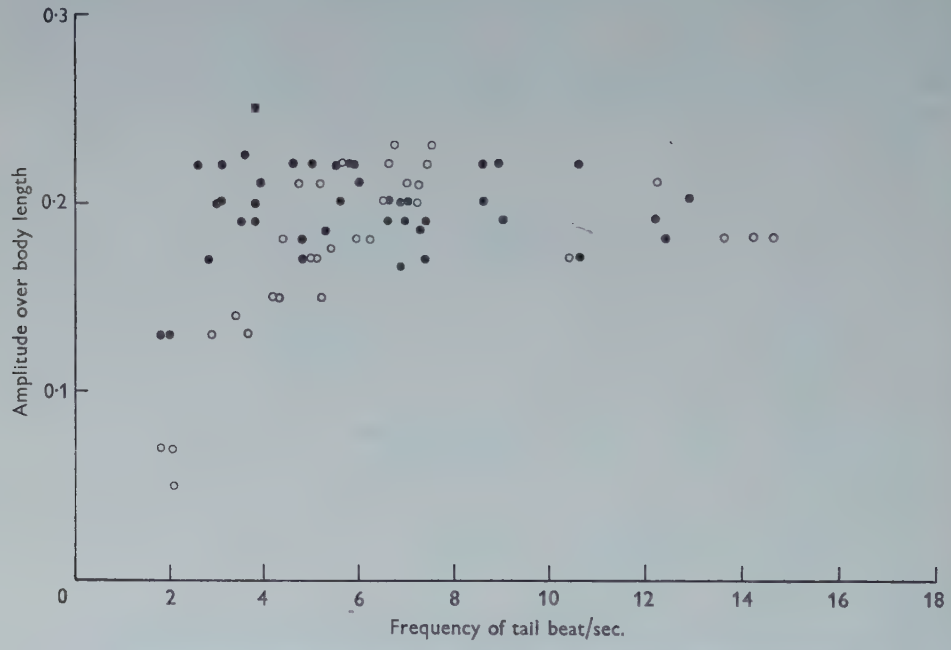

Fig. 6. Relationship between frequency and amplitude expressed non-dimensionally for two dace measuring 24·0 cm. (black circles) and 17·5 cm. (white circles).

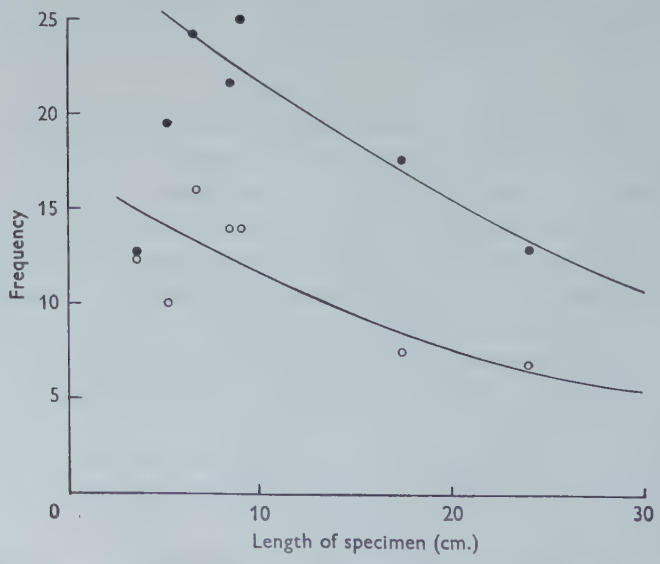

Fig. 7. Maximum recorded (black circles) and maximum sustained (white circles) frequencies for dace of different lengths. For further explanation see text.

that this is generally so, except for the smallest specimen of all. It thus appears that the larger specimens are unable to wag the tail as rapidly as the smaller ones. The implication of this in the prediction of the speed of much larger fish is considered later. The maximum frequencies recorded are rarely sustained in the wheel for any length of time. A more interesting parameter could therefore be the maximum frequency that can be sustained indefinitely. This may possibly be obtained by an examination of the distribution of recorded frequencies for each fish (i.e. as shown in Fig. 16 for a goldfish). When this is done for the 17·5 cm. dace in Fig. 3A, for example, there appears to be a discontinuity at 7·5 beats per second; while for the 9·0 cm. dace in Fig. 3C this happens at about 14·0 beats per second. It is here suggested that these discontinuities arise because of the small number of occasions upon which the maximum sustainable speed was exceeded for long enough to tempt the experimenter into taking a ciné record. Assuming that the experimenter made the same effort to obtain representative records for each size of fish it is possible tentatively to identify the discontinuity with the maximum sustainable frequency. Such values for all the specimens are also plotted in Fig. 7 (white circles) and seem to approximate to a curve at about half the maximum attainable frequency. The use of this curve in prediction is also considered later.

Summarizing, we may say that the speed of the various dace studied is dependent upon the length of the specimen and the frequency and the amplitude of the tail beat. Above about 5 beats per second the amplitude has reached a steady maximum value making the relationship between speed and frequency thereafter linear. The maximum speed attainable depends upon the maximum frequency, which decreases with increasing size of the fish.

The trout, Salmo irideus

The results obtained using four trout may now be considered. Fig. 8A–D shows the speed/frequency relationship of these specimens, measuring respectively 29·3, 23·2, 13·5 and 4·0 cm., and Fig. 8E shows speed over body length plotted against frequency for all the fish. The relationship here appears to differ slightly from that for the dace at the lowest frequencies and can most simply be represented by a straight line cutting the abscissa at a frequency of about 2 beats per second, an interpretation supported by the relationship between frequency and the distance travelled per beat over body length (graph not reproduced). This tendency for the line to cut the abscissa at a finite frequency of 2 beats per second is especially clear in the results for the 13·5 and 4·0 cm. specimens. The results for the latter, kindly provided for me by Mr T. G. Northcote, are interesting in combining two techniques. The values down to about 6 or 7 cm. per second were obtained with the small fish wheel; those below this by photographing the fish in an experimental flume through which a steady current of water was flowing. These two sets of points fall well into line and are mutually corroborative.

The dependence of speed upon amplitude is also apparent for these fish. The mean amplitude for each period of steady swimming has again been calculated, and for the 29·3, 23·2 and 13·5 cm. specimens distance travelled per beat is plotted

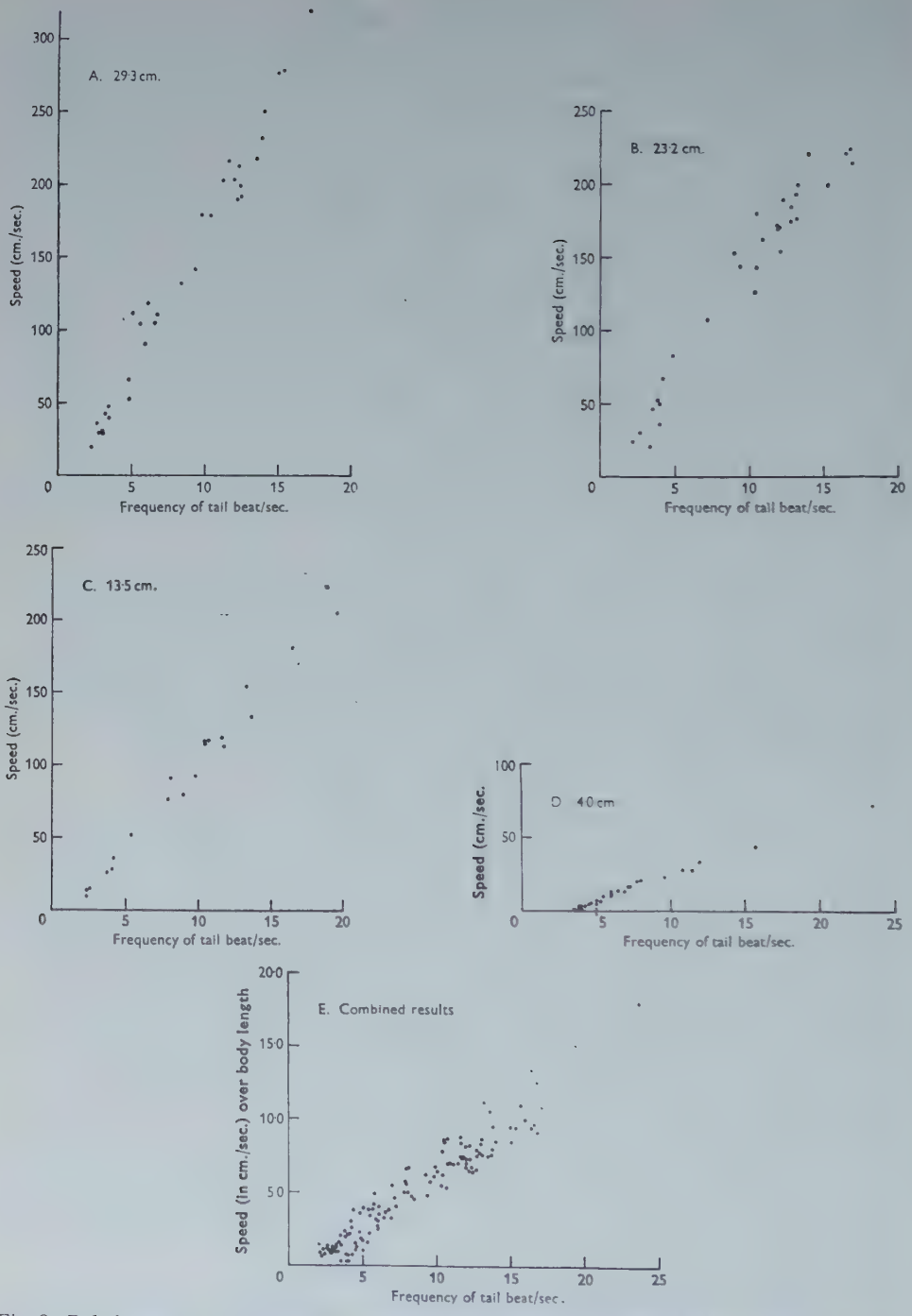

Fig. 8. Relationship between speed of swimming and frequency of beating of the tail for specimens of the trout (*Salmo irideus*). A 29·3, B 23·2, C 13·5 and D 4·0 cm. long. E shows all these results with the speed expressed non-dimensionally as body lengths per second.

against amplitude in Fig. 9. The relationship again appears linear and bears a close resemblance to that for the dace. The slope of the line is perhaps a little steeper, indicating a greater efficiency at higher amplitudes. Table 2 shows that the mean amplitude is little different, length for length, from that of the dace. The scatter in Fig. 9 shows it to be rather more variable, however, and this may be a reflexion of the greater excitability of the trout.

The relationship of amplitude over body length to frequency (Fig. 10) for the three larger fish differs from that for the dace in not showing a decrease in amplitude

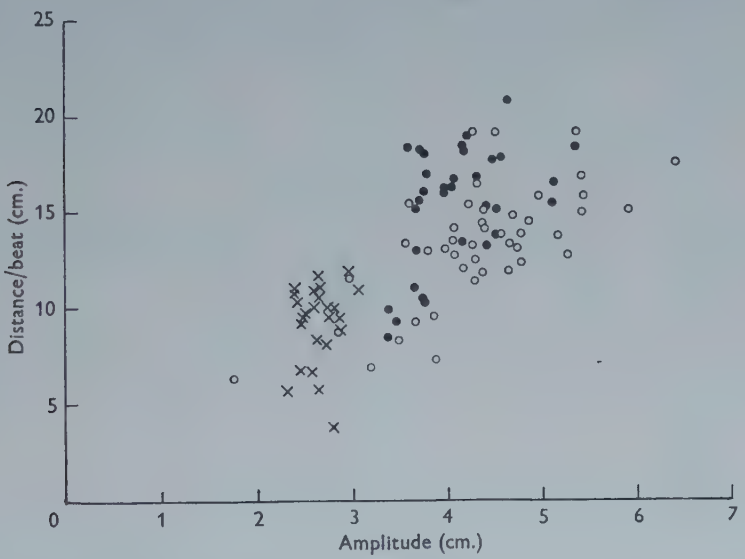

Fig. 9. Relationship between distance travelled per beat and amplitude for three trout measuring 29·3 cm. (black circles), 23·2 cm. (white circles) and 13·5 cm. (crosses).

Table 2. *Values of mean amplitude over body length at the maximum steady amplitude*

Fish	Size (cm.)	No. of observations	Mean $\frac{\text{amplitude}}{\text{body length}}$
Dace (frequencies > 5)	9·0	23	0·153
	17·5	22	0·197
	24·0	25	0·198
		Total **70**	**0·183**
Trout (frequencies > 5)	13·5	19	0·197
	23·2	29	0·197
	29·3	32	0·137
		Total **80**	**0·174**
Goldfish (frequencies > 3)	15·2	24	0·196
	22·5	13	0·212
		Total **37**	**0·202**
		Grand total **187**	Grand av. **0·183**

at lower frequencies; and this confirms the interpretation just placed upon the speed/frequency graphs. Inspection of the points for the individual fish, however, shows a slight decrease for the 23·2 cm. specimen (open circles), which the wide scatter of the other points conceals. This raises the possibility that the speed/frequency graph is not linear at the very lowest frequencies. There is also an indication of a slight decrease in amplitude at higher frequencies, especially with the 23·2 cm. fish, and this of course would have the effect of making the speed frequency graph sigmoid.

These differences from the dace are, however, trivial compared with the close resemblances. Not only are the mean *amplitudes* almost identical but also, in

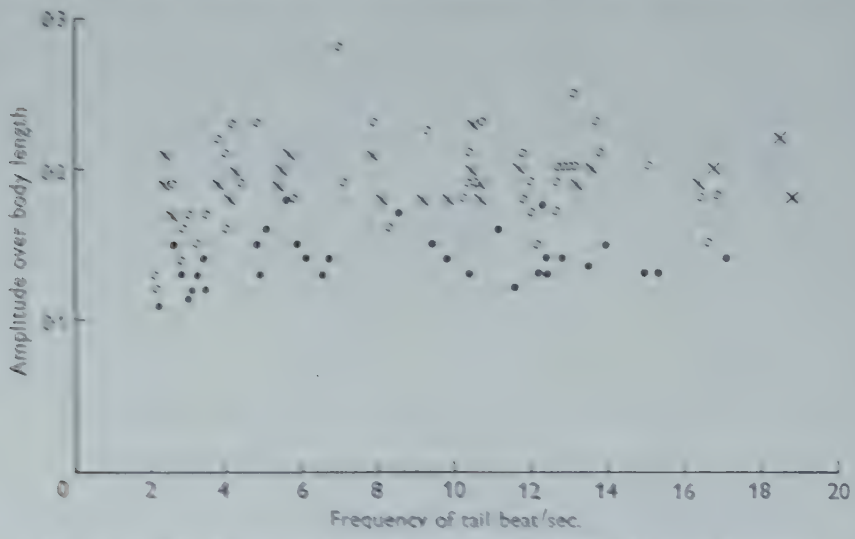

Fig. 10. Amplitude expressed non-dimensionally and plotted against frequency for the three trout shown in Fig. 9.

consequence, the mean *distances travelled per beat over body length* (Table 3). This means that, disregarding frequencies below 5 beats per second, both dace and trout have speed/frequency relationships that are virtually identical. In view of their similarity in body form some such finding might be expected, but the almost exact identity revealed is surprising.

Finally, the maximum frequencies attained by the various specimens studied are shown in Fig. 11. As with the dace a decrease in maximum frequency with increasing length is apparent; but this decrease is by no means as marked, reflecting the fact that the larger trout can reach relatively greater maximum speeds than the larger dace. The uniform distribution of recorded frequencies throughout the measured ranges in the speed/frequency graphs precludes any estimate of a maximum sustained frequency. (The disproportionate number of low measurements for the 4·0 cm. specimen derives from a deliberate attempt to record speeds as low as possible, and this graph differs in this respect from all the other records.) Indeed,

during the measurements all the trout seemed capable of sustaining even the highest frequencies and speeds for much greater periods than the dace and were consequently much easier to work with.

Table 3. *Values of mean distance travelled per beat over body length at the maximum steady amplitude*

Fish	Size (cm.)	No. of observations	Mean $\frac{\text{distance/beat}}{\text{body length}}$
Dace (frequencies > 5)	3·6	5	0·52
	5·2	21	0·63
	6·6	40	0·60
	8·5	18	0·56
	9·0	48	0·64
	17·5	22	0·67
	24·0	25	0·68
		Total **179**	**0·63**
Trout (frequencies > 5)	4·0	18	0·52
	13·5	19	0·76
	23·2	29	0·62
	29·3	25	0·58
		Total **91**	**0·62**
Goldfish (frequencies > 3)	4·6	8	0·57
	7·0	21	0·59
	9·5	30	0·54
	15·2	24	0·68
	22·5	13	0·68
		Total **96**	**0·61**
		Grand total **366**	Grand av. **0·62**

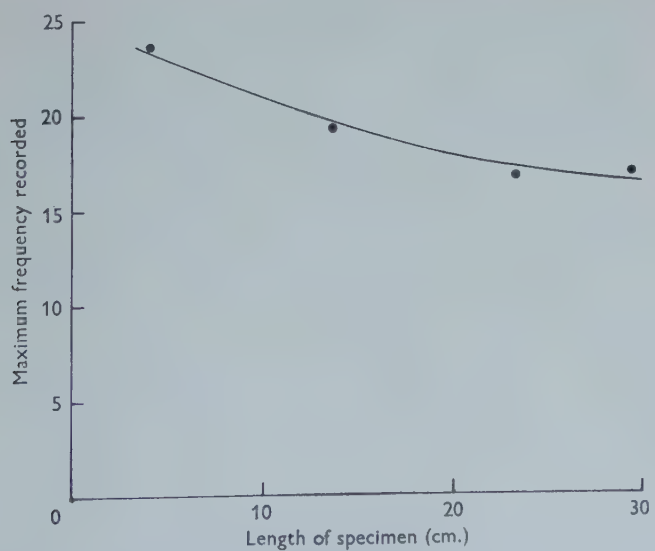

Fig. 11. Maximum recorded frequencies for the four trout shown in Fig. 8.

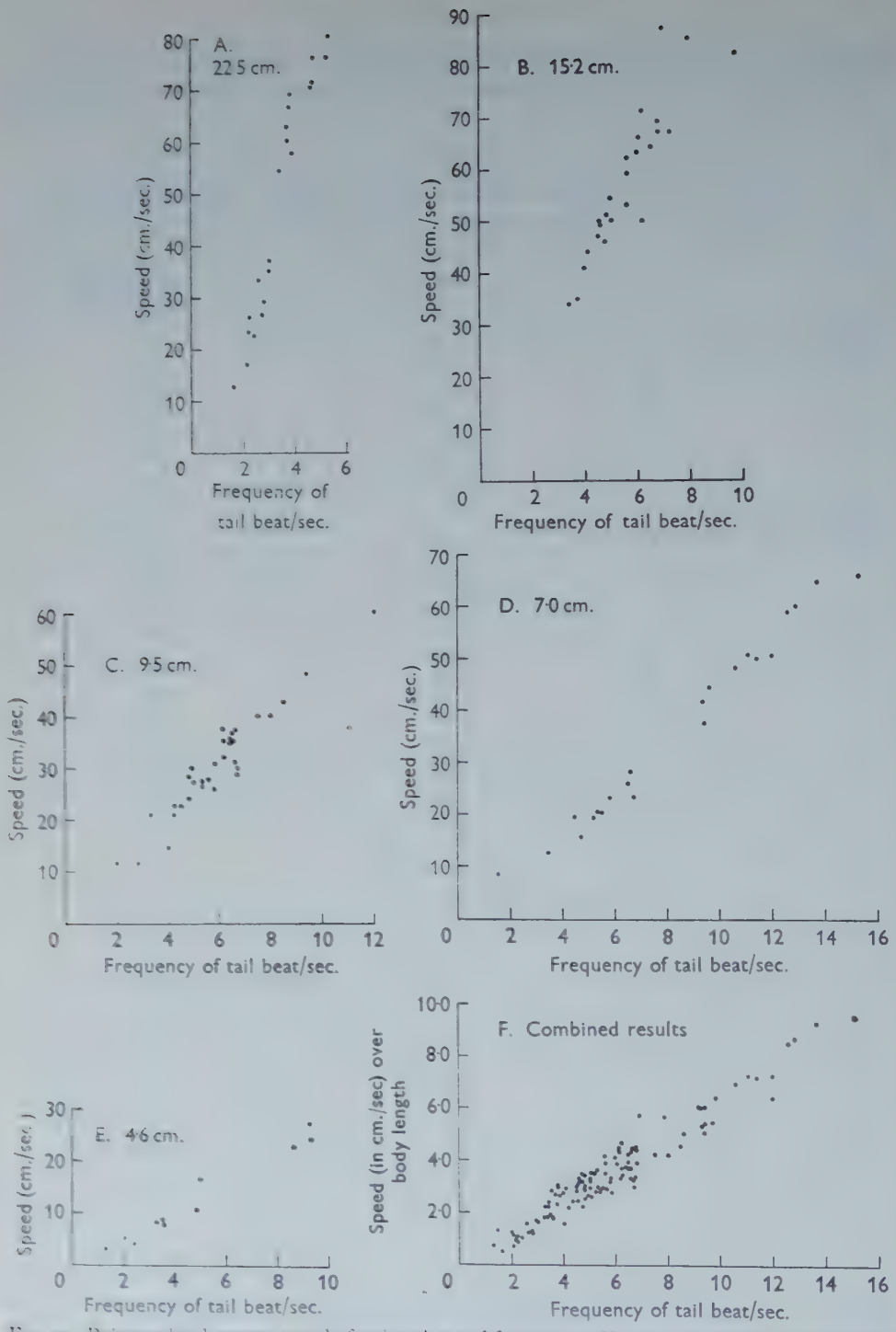

Fig. 12. Relationship between speed of swimming and frequency of beating of the tail for specimens of the goldfish (*Carassius auratus*). A 22·5, B 15·2, C 9·5, D 7·0 and E 4·6 cm. long. F shows all these results with the speed expressed non-dimensionally as body lengths per second.

Summarizing we may say that for the trout as for the dace speed is again dependent upon length, frequency and amplitude. There is little recognizable variation of amplitude with frequency, the speed/frequency relationship is entirely linear and is identical in form with that for the dace above 5 beats per second. Length for length, greater maximum frequencies and hence greater maximum speeds are attainable by the trout.

The goldfish, Carassius auratus

Graphs of speed plotted against frequency for the five goldfish studied are shown in Fig. 12A–E. The specimens range in size from 22·5 to 4·6 cm. As with the trout the relationship again appears to be linear and this is made clearer, together with a confirmation of the essential dependence upon length, in Fig. 12F, which shows speed over body length plotted against frequency. The plot of distance travelled per

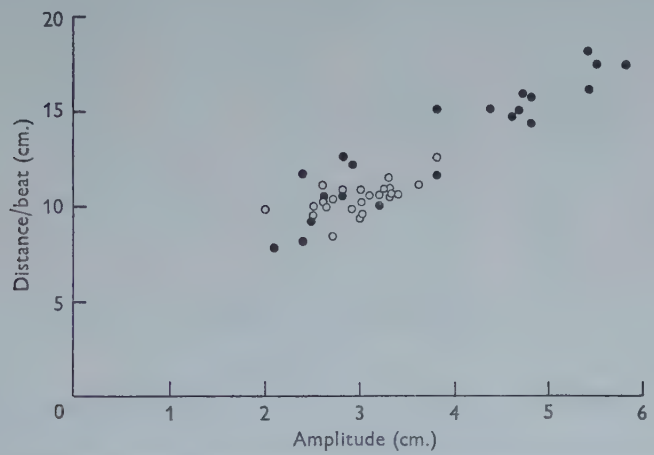

Fig. 13. Relationship between distance travelled per beat and amplitude for two goldfish measuring 22·5 cm. (black circles) and 15·2 cm. (white circles).

beat against frequency (graph not reproduced), which is more sensitive as an indicator of non-linearity at lower frequencies, suggests that there may be a very slight bend below about 3 beats per second, but this is not very clear.

The variability of amplitude and the dependence of speed upon this are even more clearly seen in the two largest goldfish. Fig. 13 shows the relationship between amplitude and distance travelled per beat for these two fish. With the 22·5 cm. specimen the relationship is linear over a range of amplitude from 2 to 6 cm. The slope of this line is almost identical with that for the dace. The range of amplitude variation for these two fish appears greater than that in most of the others, but the correlation with distance travelled per beat is much better. Calculated for frequencies greater than 3 beats per second, the mean amplitude over body length (Table 2) is a little higher than that for the dace and trout, but the distance travelled per beat over body length (Table 3) is identical.

The plot in Fig. 14 of amplitude over body length against frequency for the two fish measured reveals, especially with the 22·5 cm. specimen, what may be a slight

decrease in amplitude at low frequencies. From this it could be deduced that the speed/frequency relationship is not linear at low frequencies but resembles that for the dace. If this is so, the deviation from linearity is so slight as not to be apparent with the small number of points involved.

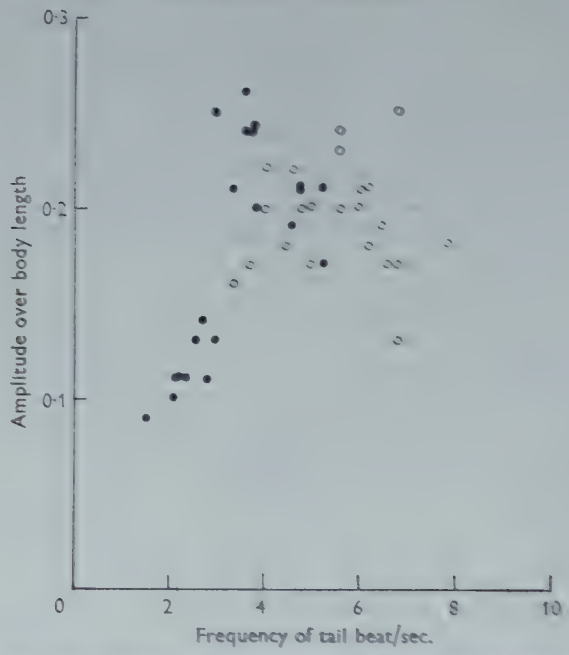

Fig. 14. Amplitude expressed non-dimensionally and plotted against frequency for the two goldfish shown in Fig. 13.

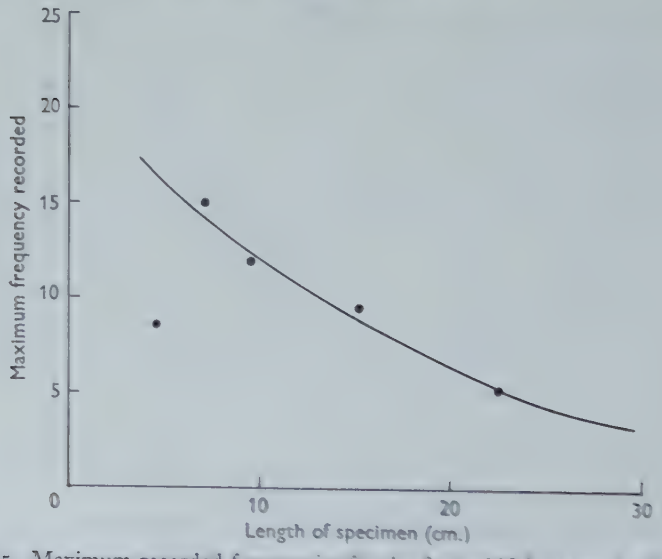

Fig. 15. Maximum recorded frequencies for the five goldfish shown in Fig. 12.

Since for the goldfish distance travelled per beat over body length is the same as for the other two genera, it follows that the linear portion of the speed/frequency graph is also the same for all. In view of the markedly different form of the goldfish body this must occasion more surprise than the resemblance between the dace and the trout. The goldfish differs from the others most obviously in the much lower maximum speed of which it appears capable, of the order of one-third of the maxima for the other two species, length for length. The identity of the speed/frequency graphs makes it clear that this limitation is imposed upon the goldfish by an inability to beat the tail more rapidly rather than, say, by inefficiency of the tail mechanism or higher resistance of the body.

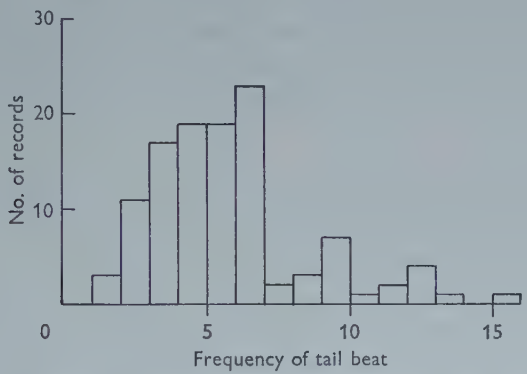

Fig. 16. Number of records at different frequencies for the five goldfish shown in Fig. 12. For further explanation see text.

The maximum frequencies recorded for the various goldfish are shown plotted against length in Fig. 15. Except for the smallest specimen, which we may presume was not at its limit, the points fall on a curve of the same general form as for the dace and the trout, but differing in that (i) the highest figure for the smallest specimens is 15 beats per second compared with about 25 for the other two species, and (ii) the lowest figure is 5 beats per second compared with 14 for the dace and 17 for the trout. These differences are reflected in the much poorer all-round performance of the goldfish. Except for the 9·5 cm. specimen the records are again fairly uniformly distributed throughout the frequency range, and it is not possible to recognize a maximum sustainable speed. Nevertheless, the impression from the experimentation remains that there probably is one and that it is fairly low, possibly corresponding to about 6 beats per second. This is supported by the histogram in Fig. 16 which shows the frequency distribution of all the records of frequency of tail beat made for the five goldfish. A limit below 7 is very clear and this would correspond to a speed of about 3·5 body lengths per second.

Summarizing, we may say that for the goldfish speed is related to length, frequency and amplitude in a manner identical with that found for both the trout and the dace. The maximum frequencies attained by the goldfish are about three-fifths of those for the trout and the dace, and the maximum speeds are correspondingly lower.

DISCUSSION

The three sets of results described have certain features in common. In all cases the direct dependence of speed at any particular frequency upon length and also upon amplitude is substantiated. Above about 5 beats per second for the dace and trout and above 3 beats per second for the goldfish the amplitude, and hence the distance moved per beat, are at a maximum. Above these frequencies the speed/frequency relationship must therefore be linear, as indeed the speed/frequency graphs show.

When distance moved per beat is plotted against amplitude for the three species studied, not only are the relationships similar but so are the absolute values. Figs. 6, 9 and 13 may all be superimposed, and the dace and goldfish points lie well within the scatter of the trout ones. This similarity is at once striking and puzzling. If the form of the body had a marked influence on the speed attained it is in these graphs that differences would be most apparent; but there is little indication of any dependence of speed upon the shape of the body. The hydrodynamical implications of this fact are yet to be considered.

If speed over body length (Figs. 3E, 8E and 12F) is plotted against frequency (discarding frequencies of less than 5 beats per second) the points, as expected, are found to lie well on a single line. This line has a slope of 1 in $1\frac{1}{3}$ and it cuts the abscissa at a frequency of $1\frac{1}{3}$. It can, therefore, be expressed conveniently by the equation $V=\frac{3}{4}\{L(f-1\frac{1}{3})\}$ or $V=\frac{1}{4}\{L(3f-4)\}$ where V is the speed in cm. per second, f the frequency in beats per second and L the length of the specimen in cm. This gives a single simple equation by means of which the speed of any specimen of the three species considered may be calculated from its length and the frequency of beating of the tail. While theoretically the equation applies only to frequencies greater than 5 beats per second, the deviation below this value is so slight as to make no material difference to the calculated speed except in the case of the smallest specimens.

At frequencies below 3–5 beats per second the exact form of the speed/frequency graph is not clear and one may be tempted to distinguish fundamental differences between the three species. However, in all cases at low frequencies the amplitude varies with frequency. This fact is especially clear with the 24·0 and 17·5 cm. dace, with the 23·2 and 13·5 cm. trout and with the 22·5 cm. goldfish. It implies that all the fish are responding similarly, although in some cases the small number of points at low frequencies and the smallness of the differences prevent this being apparent. Thus, at these low frequencies the amplitude itself is apparently related to the frequency and the speed is therefore related to the square of the frequency. This dual dependence upon frequency produces a curve which may or may not go through the origin. In the dace it probably does so, in the trout not. In the goldfish it probably goes through the origin, but becomes linear earlier than in the others, since the amplitude reaches its maximum sooner.

Preoccupation with these small differences at low frequencies must not divert our attention from the striking identity of the relationship at higher frequencies; and in considering the prediction of the maximum speeds attainable by larger fish it is

legitimate to ignore such differences. Making the assumption that the equation $V=\frac{1}{4}\{L(3f-4)\}$ is applicable to fish of any size and species we may consider how calculated values of speeds for larger fish compare with such as have already been reported in the literature. In calculating possible maximum speeds the upper limit of f remains as the controlling factor. Unfortunately, a certain amount of conjecture is involved in estimating its maximum value for bigger fish, our only evidence being the trends in Figs. 7, 11 and 15. Taking first the values for the trout in Fig. 11 it appears by extrapolation that a 100 cm. specimen (*ca.* 3 ft.) might have a maximum frequency of *ca.* 14 beats per second. Substituting in the equation above, this would give a top speed of $9{\cdot}5L$ or 950 cm. per second, that is about 20 m.p.h. This figure compares favourably with the first of Gray's (1953) calculations of $9{\cdot}2L$ and $12{\cdot}2L$ for a 3 ft. salmon assumed jumping 6 and 10 ft. out of the water. On the other hand, his photographic record giving $8{\cdot}5L$ for a 20 cm. rainbow trout is probably low, as such a specimen could easily attain a maximum frequency of 18 and a consequent speed of $12{\cdot}5L$.

Gero's (1952) value of $9{\cdot}4L$ for a 51·0 in. barracuda (130 cm.) also seems reasonable if this fish is thought to be as efficient as the trout. Its maximum frequency might be down to 13, which would give a velocity of $8{\cdot}8L$. The value given by Lane (1941) for a 60 lb. tuna of $13{\cdot}4L$ per second does however seem surprisingly high. This fish, weighing 60 lb., may be calculated as 147 cm. long and its maximum frequency on the trout scale could hardly be over 12, giving a speed of $8{\cdot}0L$. Assuming the accuracy of the estimate of speed the tuna must then be thought of as being highly efficient compared with the trout. The last very high figure is that of Denil (1937) for a 25 cm. trout reported as leaping 1 m. It would seem very unlikely that such a specimen could exceed a speed of $12{\cdot}0L$ and his value of 19·4 must be counted as exaggerated. Such reasonable confirmation of speeds of the order of 10 or $12L$ per second for larger fish emphasizes again the physiological problems involved in driving the tail sufficiently rapidly to produce such speeds for any length of time. These problems, that of drag at the body surface and the functioning of the tail as an organ of propulsion, may be considered at some later time.

These calculations have all been based on the assumption of a diminution in frequency with length, similar to that seen in the trout. The maximum frequency curve for the dace shows a much more rapid decline and any extrapolation based on it would give values substantially less. Such figures might be comparable with the low speeds given by Gero (1952) for some of the sharks.

While the dace and trout may be looked upon as fairly similar in their general behaviour, the goldfish differs fundamentally in having its maximum speed so markedly limited by the frequency of beating of the tail. Its mean maximum amplitude of beating is $0{\cdot}202L$ compared with $0{\cdot}174L$ for the trout and $0{\cdot}183L$ for the dace (Table 2). Despite this slightly higher amplitude the goldfish travels a slightly shorter distance for each beat of the tail at its maximum amplitude: the mean values for distance travelled per beat being: goldfish $0{\cdot}61L$, trout $0{\cdot}62L$ and dace $0{\cdot}63L$. These values are all well within the limits of variability for each series of fish (Table 2), and it must be assumed that the mean maximum amplitude and

hence the distance travelled per beat is the same for all three species. In the case of the goldfish this is surprising as its body shape is such as to present a greater resistance to passage through the water than in the case of the dace and the trout, which resemble each other closely. The explanation may lie in its greater tail area and the laterally flattened peduncle which may also provide greater thrust. The greater power needed to propel it the same distance per beat with a bigger tail would also impose a lower maximum frequency upon a fish of any given length.

Since the calculated values correspond reasonably well with such recorded values as exist for the speed of larger fish, we may draw the following conclusions. The speed of any fish may be calculated from the formula $V=\frac{1}{4}\{L(3f-4)\}$. The maximum values of f attainable vary according to the species; for 30 cm. specimens of dace, trout and goldfish they may be taken as 11, 17 and 3, respectively. Above this size prediction depends upon the relation between maximum frequency and length. For a trout type the decline of frequency with length seems to be gradual and values may be as high as 15 for a 60 cm. fish and 14 for a 100 cm. one. For a dace and a goldfish type corresponding values might be down to 1 or 2, and in any case with these fish the maximum frequency may not be sustained for any length of time. A maximum sustainable frequency for these fish would perhaps be about half the maximum attainable one.

There seems every likelihood that the linear relation between speed and frequency should apply to much bigger fish. It may, therefore, be possible to assess the speed of large fish not by timing, which in nature is very difficult, but simply by listening to and recording the sound of their tail beat. The development of a hydrophonic device for picking up and recording the frequency of these sounds should not be insuperably difficult and would open up the possibility of rewarding field work.

SUMMARY

1. An apparatus is described in which it is possible to study and record the continuous swimming of fish at speeds up to 20 m.p.h.

2. Records made of the swimming at different speeds of dace, trout and goldfish measuring up to 30 cm. in length are reproduced.

3. Speed at any particular frequency of tail beat is shown to be directly related to the length of the specimen, measured from the tip of the snout to the most posterior extremity of the tail.

4. Above a frequency of 5 tail beats per second speed is directly dependent upon frequency up to the maximum values recorded. The results for all sizes and species recorded may be adequately expressed by the formula $V=\frac{1}{4}\{L(3f-4)\}$, where V is the speed in cm. per sec., f is the frequency in beats per sec. and L is the body length in cm.

5. The distance travelled per beat (and hence the speed) is directly dependent upon the amplitude of the tail beat.

6. The amplitude increases with increasing frequency up to a maximum reached at about 5 beats per second. This maximum amplitude is the same for all fish tested and is about one-fifth of the body length.

7. The maximum frequency attainable decreases with increasing size of the specimen. This decrease is slight in the trout and more pronounced in the dace and goldfish. Estimation of the possible maximum frequencies of much bigger fish allows for prediction of the maximum speeds they may be able to attain. Such predicted speeds are in accord with the few measurements that have been made and are of the order of 10 body lengths per second up to a size of 1 m.

I am indebted to Professor Sir James Gray, F.R.S., for his continued helpful interest in this work; to Dr R. H. J. Brown who shared in the construction of the large wheel; and to Messrs Watts, Watts and Company Ltd., the British Steamship Company Ltd. and the British Thomson-Houston Company Ltd. for their generous gifts which made possible the building of the large apparatus.

REFERENCES

Bainbridge, R. & Brown, R. H. J. (1958). An apparatus for the study of the locomotion of fish. *J. Exp. Biol.* **35**, 134–7.

Dahl, K. & Sømme, S. (1936). Experiments in salmon marking in Norway, 1935. *Skr. norske Vidensk-Akad.* I. Matem. Naturvid Klasse 1935. No. **12**, 1–27.

Davidson, M. Viola (1949). Salmon and eel movement in constant circular current. *J. Fish. Res. Bd. Can.* **7**, 432–48.

Denil, G. (1937). La mécanique du poisson de rivière. Chapitre X. Les capacités mécaniques de la truite et du saumon. *Ann. Trav. publ. Belg.* **38**, 412–33.

Fry, F. E. J. & Hart, J. S. (1948). Cruising speed of goldfish in relation to water temperature. *J. Fish. Res. Bd. Can.* **8**, 169–75.

Gero, D. R. (1952). The hydrodynamic aspects of fish propulsion. *Amer. Mus. Novit.* no. 1601, 1–32.

Gray, J. (1953). The locomotion of fishes. In *Essays in Marine Biology*, 1–16. Edinburgh: Oliver and Boyd.

Hardy, A. C. & Bainbridge, R. (1954). Experimental observations on the vertical migrations of plankton animals. *J. Mar. Biol. Ass. U.K.* **33**, 409–48.

Hecht, S. (1916). Form and growth in fishes. *J. Morph.* **27**, 379–400.

Lane, F. W. (1941). How fast do fish swim? *Ctry Life, Lond.*, 534–5.

Magnan, A. (1930). Les caractéristiques géométriques et physiques des poissons. *Ann. Sci. nat.* **13**, 355.

Radcliffe, R. W. (1950). The effect of fin clipping on the cruising speed of goldfish and coho salmon fry. *J. Fish. Res. Bd. Can.* **8** (2), 67–72.

Regnard, P. (1893). Sur un dispositif qui permet de mesurer la vitesse de translation d'un poisson se mouvant dans l'eau. *C.R. Soc. Biol., Paris*, Ser. 9, **5**, 81–3.

Stringham, E. (1924). The maximum speed of fresh water fishes. *Amer. Nat.* **58**, 156–61.

Wales, J. H. (1950). Swimming speed of the western sucker *Catostomus occidentalis* Ayres. *Calif. Fish Game*, **36**, 433–4.

AN APPARATUS FOR THE STUDY OF THE LOCOMOTION OF FISH

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(*Received* 14 *June* 1957)

In studies on the swimming of fish using the small 'fish wheel' (Bainbridge, 1958) it became clear that to enable the research on swimming speeds to be extended to larger specimens and to give scope for the investigation of other problems, a larger apparatus was needed. With the generous support of Messrs Watts, Watts and Company Ltd., The British Steamship Company Ltd., and the British Thomson-Houston Company Ltd., the development of this large wheel became possible. The construction of the wheel itself was undertaken in the Zoological Department, Cambridge, while the electric drive was supplied by the British Thomson-Houston Company.

The basic principle, following Hardy & Bainbridge (1954), is simple; the fish swims in the water-filled rim of a large wheel which is rotated at a speed equal to that of the fish, but in the opposite direction. The animal thereby remaining stationary relative to the observer.

The wheel (Fig. 1), which rotates about a vertical axis, is 7 ft. 6 in. in diameter, and has a hollow Perspex rim of rectangular section. This rim is divided into three sections by radial plates or doors, which can be withdrawn into pockets projecting towards the centre from its inner wall. The movement of each door is effected by a double-acting cylinder fed with compressed air at 25 lb./in.^{2} from a central distributing valve, which is so arranged that each door opens at a fixed point relative to the mounting and closes 120° away from this point. There is, therefore, always at least one door closed, and the water is constrained to move at the same speed as the wheel.

The cross-section of the water space is 6 in. wide and $7\frac{1}{2}$ in. high. The top and the bottom are made from $\frac{1}{2}$ in. thick Perspex and are grooved to take the heat-formed side-walls. The bottom projects inwards towards the centre forming a flange 7 in. wide, while four 15 in. long removable sections in the top allow for access.

The centre pivot carries a 2 ft. diameter steel disk, to which six channel-section light alloy spokes are attached; each of these is bolted to the Perspex flange by six bolts. The attachment load is distributed by glass-fibre sheets cemented to the Perspex around the bolt holes. The pivot is required only to locate the wheel and withstand the driving torque; the weight of the rim (about 5 cwt.) being carried on eight roller-bearing wheels with hard rubber tyres. These are placed to bear on the flange just inside the inner wall of the tubular rim, thereby leaving the under surface free for observation (Fig. 2).

The complete wheel is mounted on a 6 ft. square angle-iron framework, which is fastened to the floor on one side with hinge-joints, while the opposite side fits into sockets. This enables the whole structure to be tilted about 5° by raising one side on a hydraulic jack. This tilting is essential to prevent large air bubbles forming under the top of the rim when it is being filled with water, as well as being useful when it is being emptied.

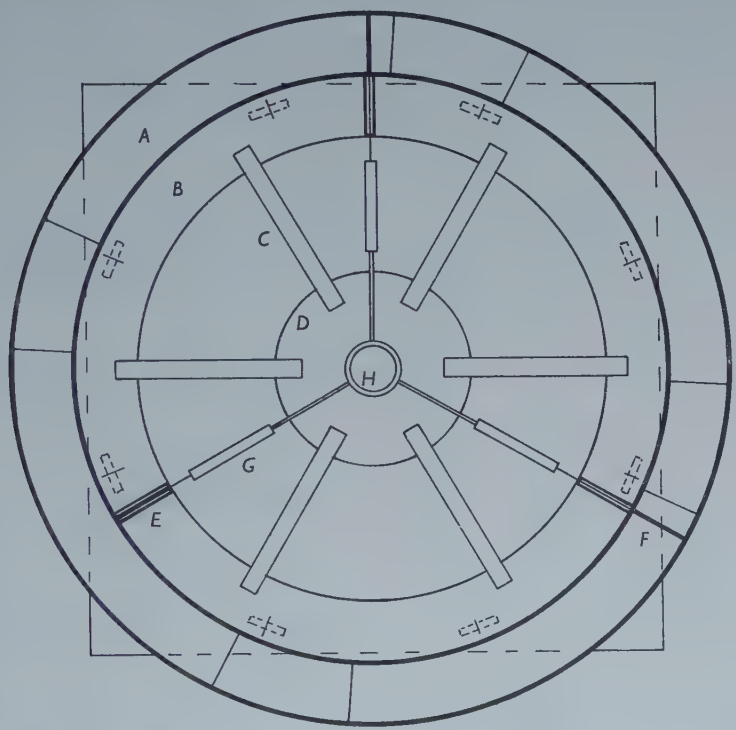

Fig. 1. Diagrammatic view of fish wheel from above showing: *A*, tubular Perspex rim; *B*, inner flange; *C*, spokes; *D*, central steel disk; *E*, sliding door withdrawn into pocket; *F*, sliding door closed across water space; *G*, air cylinder; *H*, distributing valve.

The rotation is effected by a 5 h.p. d.c. motor with 10 h.p. overload rating, driving the vertical central shaft through a reversible worm gear box; between this box and the wheel is a rubber flexible coupling to absorb the shock loads when the drive is reversed. Speed control is carried out by an electronic control unit which operates as follows. The operator's control alters a voltage which is compared in the electronic unit with the output of a small d.c. generator driven by the main motor. The resulting difference voltage is caused to control, both in magnitude and sign, the excitation of a motor-generator set feeding the driving motor. The maximum permitted acceleration can be adjusted by controls which limit the error signal to be accepted. A complication arises from the fact that the efficiency of the worm drive is higher when the motor is accelerating the wheel than it is when the wheel is decelerating against the braking effect of the reversed motor. This would make

the deceleration much more rapid than the acceleration at any particular control setting; and should the acceleration be adequate the corresponding deceleration would impose too great a load on the mechanism. This difficulty is overcome by biasing the set levels asymmetrically about zero, and by reversing the bias as the direction of rotation changes. This is achieved by a changeover switch operated by the motor through a slipping magnetic drive.

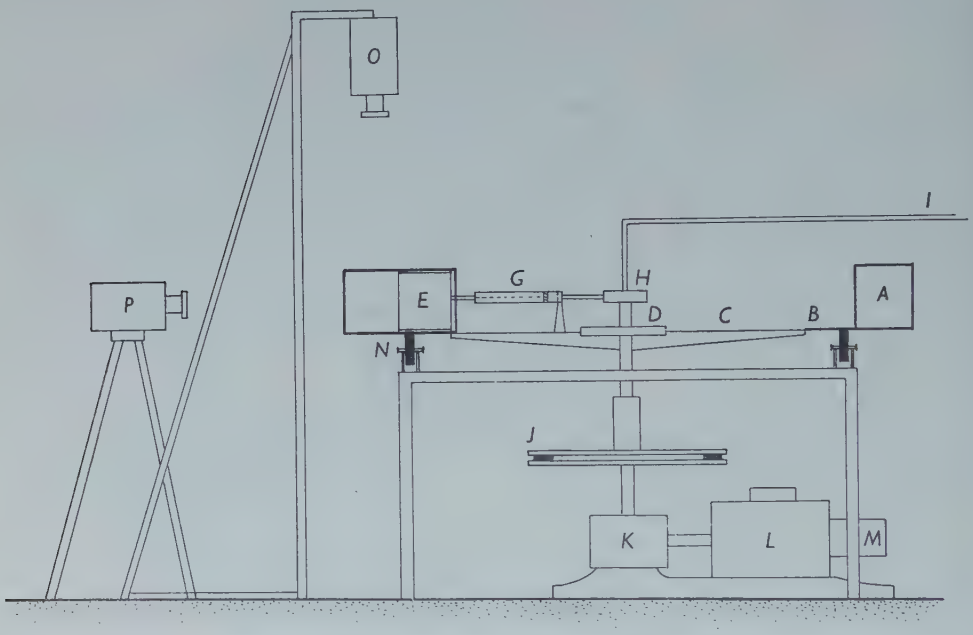

Fig. 2. Diagrammatic view of fish wheel from the side showing: *A*, tubular Perspex rim; *B*, inner flange; *C*, spoke; *D*, central steel disk; *E*, door; *G*, air cylinder; *H*, distributing valve; *I*, compressed air supply; *J*, rubber shock damper; *K*, worm drive; *L*, motor; *M*, speed control generator; *N*, roller race supporting wheel; *O*, vertical camera; *P*, horizontal camera.

The operator's control panel is carried on a small stand and connected by a flexible cable to the main unit so that it can be placed at any convenient point. This panel carries the speed control, stop and start buttons, and a speed range switch with four positions: 0–20, 0–60, 0–200 and 0–600 cm./sec. as measured at the mean radius of the rim. This switch also selects the correct range on a 6 in. Cirscale speed meter whose dial is calibrated in centimetres per second.

Photographic records of the swimming fish are obtained by two electrically driven 35 mm. Vinten cameras, one on a horizontal stand, and the other arranged to photograph either through the top or the bottom of the wheel. The vertical camera is also arranged to photograph a reduced image of the speed meter. The cameras are coupled by a flexible shaft with phasing control, so that their exposures take place at the same time. The camera speed can be 16, 32 or 48 pictures per second, and is held constant by an electronic circuit which varies the voltage of the supply

to the camera motors and is itself controlled by an electro-mechanical integrator driven by one of the cameras.

Lighting is provided by four photoflood lamps fitted with small series resistances which are automatically shorted out only when the cameras are running, thus greatly increasing the life of the lamps which must be kept burning to avoid disturbing the animal.

Finally, it has been found necessary to have a water storage tank in the room with the apparatus to allow the water to reach the same temperature as the wheel; water direct from the supply mains deposits a multitude of small air bubbles on the Perspex and seriously interferes with photography.

REFERENCES

BAINBRIDGE, R. (1958). The speed of swimming of fish as related to size and to the frequency and amplitude of the tail beat. *J. Exp. Biol.* **35**, 109–33.

HARDY, A. C. & BAINBRIDGE, R. (1954). Experimental observations on the vertical migrations of plankton animals. *J. Mar. Biol. Ass., U.K.*, **33**, 409–48.

EMBRYONIC MORTALITY IN RELATION TO OVULATION RATE IN THE HOUSE MOUSE

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INTRODUCTION

An inverse relationship between the numbers of foetuses in the two horns of the uterus in the house mouse during the last week of gestation has been reported several times (Hollander & Strong, 1950; Runner, 1951). We recently found a significant negative correlation between the two ovaries in respect of the number of corpora lutea present at 18 days of gestation. In respect of implantation sites and live embryos at 18 days, however, the correlations were much less strongly negative and were non-significant. This reduction in the strength of the correlation points to a differential loss of eggs or of embryos, the horn receiving the greater number of eggs suffering a proportionately greater loss. We have accordingly re-investigated these correlations and looked for direct evidence of a differential loss. We have found an increasing proportional loss of eggs related to the number shed within a horn, and have shown that it occurs after fertilization but before implantation. The results are reported below.

RESULTS

Two series of observations were made. In the first, pregnant female mice of heterogeneous origin were dissected at 18 days of gestation. The numbers of corpora lutea were counted as a measure of the number of eggs shed from each ovary. The numbers of implantation sites and of live embryos in each uterine horn were also counted. The correlation between sides within mice for corpora lutea was $-0{\cdot}436$ ($P<0{\cdot}001$). In agreement with our previous findings, the numbers of implantation sites and live embryos showed lower negative correlations between sides than the number of eggs shed. The correlation between sides for implantation sites was $-0{\cdot}300$ ($P<0{\cdot}02$) and for live embryos $-0{\cdot}111$ (N.S.).

Further analysis revealed that the loss of eggs is affected by the number of eggs shed into the horn. Horns with a larger number of eggs suffer a proportionately greater loss. Thus, mice with an extreme distribution of eggs between sides will contribute largely to the negative covariance in corpora lutea counts, but as the two horns are affected by differential loss the negative correlation is greatly reduced by the time of implantation.

The distribution of loss of eggs and implanted embryos within uterine horns and

Table 1. *Distribution of amount and time of loss of eggs*

Corpora lutea per ovary	No. of ovaries examined	Total corpora lutea	Fraction of corpora lutea not accounted for by implants	Loss of implants up to 18 days of gestation		Fraction of corpora lutea not accounted for by live embryos at 18 days of gestation
				As a fraction of eggs shed	As a fraction of implants	
A. Within uterine horns						
1	0	0	0	0	0	0
2	6	12	0·083	0	0	0·083
3	10	30	0·066	0·066	0·071	0·133
4	24	96	0·135	0·063	0·072	0·198
5	26	130	0·085	0·092	0·101	0·177
6	28	168	0·137	0·083	0·097	0·220
7	20	140	0·171	0·029	0·034	0·200
8	9	72	0·278	0·042	0·056	0·319
9	7	63	0·254	0·222	0·298	0·476
10	2	20	0·150	0	0	0·150
Total	132	731	0·155	0·075	0·089	0·230
B. Within mice						
Corpora lutea per mouse	No. of mice examined					
8	6	48	0·125	0·104	0·119	0·229
9	12	108	0·074	0·074	0·080	0·148
10	6	60	0·133	0·117	0·135	0·250
11	15	165	0·158	0·060	0·072	0·218
12	11	132	0·106	0·053	0·059	0·159
13	10	130	0·169	0·062	0·074	0·231
14	3	42	0·167	0·119	0·143	0·286
15	2	30	0·633	0·133	0·364	0·767
16	1	16	0·188	0·062	0·077	0·250
Total	66	731	0·155	0·075	0·089	0·230

Table 2. χ^2 *analysis of loss of eggs*

Source of variation	D.F.	χ^2	M.S.	F	P
A. Within uterine horns					
(1) Eggs lost up to implantation					
Linear trend	1	13·166	13·166	10·206	0·02
Deviations from linear trend	7	9·028	1·290	8·354	0·01
Heterogeneity of loss within corpora lutea groups	123	193·885	1·576		
(2) Eggs not accounted for by live embryos at 18 days					
Linear trend	1	14·937	14·937	5·888	0·05
Deviations from linear trend	7	17·760	2·537	9·215	0·01
Heterogeneity of loss within corpora lutea groups	123	199·430	1·621		
B. Within mice					
(1) Eggs lost up to implantation					
Linear trend	1	18·938	18·938	2·571	N.S.
Deviations from linear trend	7	51·565	7·366		N.S.
(2) Eggs not accounted for by live embryos at 18 days					
Linear trend	1	13·501	13·501	1·836	N.S.
Deviations from linear trend	7	51·485	7·355		

within mice is shown in Table 1. The χ^2 analyses for linear trend of proportion lost at two stages of gestation are given in Table 2. Only the linear trends of loss within horns up to implantation and for total loss up to 18 days of gestation are significant. The loss within horns between implantation and 18 days of gestation is very irregular and does not seem to be related either to the number of eggs shed into the horn or to the number of eggs implanted. We conclude therefore that before implantation there is positive linear trend of loss of eggs with increasing numbers shed per horn. This conclusion leads us to expect to find a similar increasing proportional loss when the data are analysed on a within-mouse basis. Our data does suggest such a trend, though statistical analysis reveals this to be non-significant.

Table 3. *Fraction of eggs not fertilized in relation to number shed per ovary*

Eggs shed per ovary	No. of Fallopian tubes examined	Total no. of eggs	Fraction not fertilized
1	2	2	0·500
2	7	14	0·429
3	7	21	0·048
4	12	48	0·125
5	15	75	0·173
6	11	66	0·030
7	13	91	0·132
8	8	64	0·125
9	1	9	0·000
10	5	50	0·080
11	—	—	—
12	1	12	0·250
Total	82	452	0·126

The differential loss between horns up to implantation may be due either to a lack of fertilization or to a failure to implant, and a second series of observations was made to solve this problem. Females of heterogeneous origin, similar to those used in the first series of observations, were put singly with a male between 5.0 and 5.30 p.m. and examined the following day between 9.0 and 10.0 a.m. for vaginal plugs. Those which had mated were killed between 7.0 and 10.0 p.m. and the eggs in each Fallopian tube were extracted. From the findings of Snell, Fekete, Hummel & Law (1940), Snell, Hummel & Abelmann (1944) and Braden & Austin (1954) we considered that the majority of fertilized eggs would at that time be in the pronucleate stage. The eggs were examined by phase-contrast microscope according to the method described by Austin & Smiles (1948). The numbers of fertilized and non-fertilized eggs in each tube were counted. Judgement as to whether eggs were fertilized or not was based on the description by Austin (1951) of the formation of the pronuclei in the rat egg.

The correlation between ovaries in the numbers of eggs shed in a mouse was found to be $-0{\cdot}528$ (P 0·001), which is in good agreement with the similar correlation for corpora lutea counts. The number of eggs not fertilized per tube in relation to the total eggs shed per ovary are shown in Table 3. In marked contrast to the

implantation data, no regular trend of loss is apparent in this case. The data has also been analysed on a within-mouse basis and as expected no trend is shown. These results indicate that fertilization rate is not related to the number of eggs shed per ovary or per mouse and that it does not normally limit litter size in the mouse.

DISCUSSION

Our observations indicate that the loss of implanted eggs up to 18 days of gestation does not vary with the number of implantations in a horn, but that as the number of eggs shed into a uterine horn increases the probability of each individual egg implanting decreases. The fertilization rate is not related to the number of eggs in the horn, and therefore the factor or factors causing the variation in implantation rate must be operating on fertilized eggs or on pre-implantation embryos.

Our results and conclusions do not entirely agree with those of other workers. Danforth & de Aberle (1928) and McLaren & Michie (1956) found no correlation between the two horns for the number of implantations. As mentioned earlier other authors have reported significant negative correlations, and therefore we can only attribute the inconsistency of the results reported to heterogeneity between mice used at different laboratories.

The differential loss of eggs in our data could be explained if trans-uterine migration of eggs had occurred in many of our mice. Such migration is known to occur in rodents (Runner, 1951; Boyd & Hamilton, 1952; Young, 1953; McLaren & Michie, 1954), but these reports suggest that its frequency is very low. For this reason we have dismissed migration as an explanation of our results.

Previous work and ideas as to the causes of pre-implantational loss have been reviewed by Hammond (1952), but it is impossible to decide from our present experimental evidence which if any of the causes are applicable to our findings. Some useful conclusions may be made, however, by comparing our results with those of McLaren & Michie (1956).

From the results of an experiment, in which they transferred varying numbers of $3\frac{1}{2}$-day-old blastocysts from donor mice to normally mated recipient mice $2\frac{1}{2}$ days pregnant, they concluded that 'although we have found no limit to the number of eggs which can implant in a single uterine horn, we are beginning to approach a limit to the number of implantations which a single horn can keep alive'. In the data reported here the post-implantational loss was irregular and not proportionately related to the number of implants in the horn. The reason for this apparent difference is fairly easily found. McLaren & Michie (1956) consider that in their material the limit to the number of implantations which remain alive to 16 days of gestation may possibly be due to 'insufficiency of corpora lutea to supply the progesterone requirements of the excessive number of implantations'. In all animals included in our data, presented here, there were at least as many corpora lutea in the ovary as implantations in the horn to which it corresponded, and consequently the progesterone supply is much less likely to have been insufficient. It is possible, however, that the total number of implants per mouse surviving to birth is limited

by the level of some substance circulating in the maternal blood supply—a hypothesis favoured by Runner (1951) and by Hammond (1952).

McLaren & Michie (1956) found in their experiment that the number of successful implantations, from donor and recipient sources combined, rises linearly by increments of 0·2 for each additional egg injected. Again, this conclusion seems to conflict with our results of an increasing proportional loss of eggs up to implantation. However, the discrepancy may not be so serious as it first appears. If indeed there is no increase in the fractional loss in McLaren & Michie's data, then it seems highly probable that the increased proportionate mortality in our material occurred very soon after fertilization, in fact between fertilization itself and the stage at which the blastocysts were removed by McLaren & Michie for transplantation.

If this suggestion is correct we have a more accurate estimate of the time interval during which the differential loss of eggs takes place, and this knowledge might prove useful in further elucidation of causes of pre-implantational loss.

SUMMARY

1. Two series of observations were made to determine the time and amount of loss of eggs in relation to the number shed per ovary and per mouse.

2. The correlations between sides within mice for eggs shed was −0·528, for corpora lutea counts −0·436, for implantations −0·300, and for live embryos −0·111.

3. A positive linear trend of loss of fertilized eggs with increasing numbers of eggs per uterine horn has been shown to occur before implantation.

4. Possible causative mechanisms for the loss are discussed in relation to observations on embryonic mortality previously reported by other workers.

We wish to express our gratitude to Prof. C. H. Waddington for laboratory facilities, to Dr D. S. Falconer for much helpful criticism and advice and to Dr B. Woolf for statistical advice. J. C. Bowman gratefully acknowledges financial support from the Agricultural Research Council.

REFERENCES

Austin, C. R. (1951). The formation, growth, and conjugation of the pronuclei in the rat egg. *J. R. Micr. Soc.* **71**, 295–306.

Austin, C. R. & Smiles, J. (1948). Phase-contrast microscopy in the study of fertilization and early development of the rat egg. *J. R. Micr. Soc.* **68**, 13–19.

Boyd, J. D. & Hamilton, W. J. (1952). Marshall's *Physiology of Reproduction*, Vol. II, chap. 14. London: Longmans.

Braden, A. W. H. & Austin, C. R. (1954). Fertilisation of the mouse egg and the effect of delayed coitus and of hot shock treatment. *Aust. J. Biol. Sci.* **7**, 552–65.

Danforth, C. H. & de Aberle, S. B. (1928). The functional interrelation of the ovaries as indicated by the distribution of foetuses in mouse uteri. *Amer. J. Anat.* **41**, 65–74.

Hammond, J. (1952). Marshall's *Physiology of Reproduction*, vol. II, chap. 21. London: Longmans.

Hollander, W. F. & Strong, L. C. (1950). Intra-uterine mortality and placental fusions in the mouse. *J. Exp. Zool.* **115**, 131–47.

McLaren, A. & Michie, D. (1954). Transmigration of unborn mice. *Nature, Lond.*, **174**, 844.

McLaren, A. & Michie, D. (1956). Studies on the transfer of fertilised mouse eggs to uterine foster mothers. *J. Exp. Biol.* **33**, 394–416.

RUNNER, M. N. (1951). Differentiation of intrinsic and maternal factors governing intra-uterine survival of mammalian young. *J. Exp. Zool.* **116**, 1–20.

SNELL, G. D., FEKETE, E., HUMMEL, K. P. & LAW, L. W. (1940). Relation of mating ovulation and the oestrus smear in the house mouse to time of day. *Anat. Rec.* **76**, 39–54.

SNELL, D., HUMMEL, K. P. & ABELMANN, W. H. (1944). A technique for the artificial insemination of mice. *Anat. Rec.* **90**, 243–53.

YOUNG, A. (1953). Transuterine ('Internal') migration of fertilised ova in the albino rat. *Proc. Roy. Soc.* Edinb. B, **65**, 106–16.

THE GROWTH AND DEVELOPMENT OF MICE IN THREE CLIMATIC ENVIRONMENTS

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INTRODUCTION

In discussions of the control of variability in laboratory animals it has been suggested (e.g. Michie, 1955) that adverse environmental conditions, even if uniformly adverse, tend of themselves to increase phenotypic variability. In order to obtain direct evidence to test this view, we raised three groups of mice from birth to 4 weeks of age under uniformly cold, temperate and hot conditions, respectively. The main object was to compare the amounts of variation in respect of body weight manifested by the three groups, but a quantity of information was at the same time amassed on rates of mortality, growth and development as related to sex, litter size and climatic environment. We present this information below, together with an outline of some of the biometrical methods made necessary by the complicating effects of the dependence of litter size upon environmental conditions. We leave examination of the question of phenotypic variability to a later paper (Michie, McLaren, Ashoub & Biggers, 1958).

THE EXPERIMENT

Three constant temperature rooms were used, maintained at temperatures of approximately 28, 21 and 5° C. Daily readings of temperature and relative humidity were taken in each room by means of a whirling hygrometer. Table 1 gives the

Table 1. *Conditions of temperature* (° C) *and relative humidity in the three rooms*

	Hot		Temperate		Cold	
	Tempera-ture	Relative humidity	Tempera-ture	Relative humidity	Tempera-ture	Relative humidity
Mean	28·09	89·87	21·00	69·03	4·78	77·48
S.D.	0·79	2·52	0·81	7·75	0·59	4·39
Range	26·4–30·6	81–94	19·7–23·1	55–85	3·6–5·8	61–84

means, standard deviations and ranges of these readings for the period of the experiment. In addition, the temperature was continuously recorded in all three rooms, and in the cold room two maximum-minimum thermometers placed one

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at either end of the room were read daily. Relative humidity was continuously recorded in the hot and temperate rooms throughout the experiment. In the hot and temperate rooms the air was circulated by a fan; in the temperate room the fan gave trouble, and had to be replaced by a faster moving fan at a stage in the experiment when the litters in the room were between 2 and 3 weeks of age. In the cold room the air was intermittently stirred by a fan. In the hot room the humidity was kept high and constant by placing trays of water in front of the fan.

Nulliparous female mice of Theiler's Original strain (TO strain), judged by external appearance to be pregnant, were obtained from the National Institute for Medical Research, Mill Hill, where they had been kept at a temperature of 20–21° C. The average stage of pregnancy was about 12½ days. By the use of a table of random numbers 30 females were allotted to the hot room, 29 to the cold room, and 20 to the temperate room. Those destined for the cold room were left overnight in a cool room (10–15·5° C). The mice were kept singly in aluminium cages with open wire tops, and each received the same weighed amount of sawdust and cotton wool for bedding. The mice were fed *ad lib* on a standard pellet diet (M.R.C. diet no. 41).

The cages were examined each morning for births. The young mice were weighed individually on the day the litter was found and at weekly intervals for 4 weeks thereafter. Mice under 10 g. were weighed to 0·01 of a g.; mice of 10 g. and over to 0·1 of a g. At the second weighing (1 week of age) the mice were sexed; at the third weighing (2 weeks of age) a note was made of whether or not the eyes were open, and at the final weighing (4 weeks of age) a note was made of whether or not the vaginas of the females were patent.

THE EXPERIMENTAL GENERATION

Twenty-four litters were born in the hot room, twenty in the cold room, and eighteen in the temperate room. The average litter sizes at birth (live + dead) were 7·04, 7·25 and 8·06, respectively. The litter sizes in the hot and cold rooms proved to be more variable than those in the temperate room, significantly so in the case of the cold. This suggests that the small average litter sizes in the extreme environments were a reflexion of increased prenatal mortality rather than an accident of sampling. This increase in prenatal mortality has been confirmed in a later experiment, and agrees with the work of Barnett & Manly (1956), who found that the average litter size of females from the C57BL inbred strain was strikingly reduced at −3° C. as compared with 21 or 10° C.

Certain litters and certain individual mice were omitted from the calculations on body weight for the following reasons.

(1) If a female stopped lactating, with the result that the litter actually decreased in mean weight in the course of a week, or if a water bottle flooded a cage, all weights of the litter subsequent to the cessation of lactation or to the flood were omitted.

(2) Because of the very humid atmosphere in the hot room, some of the newborn mice stuck to the cotton-wool bedding and were thereby injured. Such mice were killed.

(3) Occasionally mice were so stunted in growth that their weights fell outside the normal distribution of the rest of the litter. If such 'runts' or outliers are included in the statistical analysis, they can spuriously inflate the estimates of variability and invalidate tests of significance with respect to means. A test for outliers based upon the method of Dixon (1953) was therefore applied to all litters, and any outlying values were excluded from the calculations.

Table 2. *Mean live litter size in the three environments*

	Weeks after birth				
	0	1	2	3	4
Hot	5·80	5·45	5·30	4·95	4·95
Temperate	8·00	7·89	7·89	7·83	7·83
Cold	6·89	5·63	5·63	5·63	5·25

Table 2, which shows the mean live litter sizes for each week in each of the three rooms, illustrates the natural mortality occurring during the course of the experiment. For the purposes of this table runts have been included; litters which suffered flooding have been omitted altogether, and newborn mice damaged by sticking to the bedding in the hot room have been omitted, thus underestimating the mean litter size in the hot room throughout. Mortality was greater in the extreme environments than in the temperate, and greater in the cold room than in the hot. In the cold room most of the deaths occurred during the first week of life; moreover, the deaths of three litters *in toto* during the first week can be added to the natural mortality indicated in Table 2. The number of litters and the number of mice of each sex actually available for analysis at each week is shown in Table 3.

Table 3. *Numbers of litters and mice used in analysis of body-weight data*

		Weeks after birth				
		0	1	2	3	4
Hot	No. of litters	22	22	19	19	19
	No. of females	145	56	46	46	46
	No. of males		61	50	50	47
Temperate	No. of litters	18	18	18	18	18
	No. of females	139	65	69	68	67
	No. of males		68	70	69	68
Cold	No. of litters	20	16	16	15	15
	No. of females	131	44	45	39	40
	No. of males		45	45	37	38

RESULTS AND THEIR ANALYSIS

Biometrical aspects

Scale. It is essential to decide at the outset on what scale we are to express body weight. One of the most important factors which affects this choice is that the various influences on the mean should act additively. There is evidence that in the case of growing mice the logarithmic scale satisfies this condition. Thus, Falconer (1953), in a long-term selection experiment for high and low 6-week weights, found that genetic differences act additively on the logarithmic scale.

The logarithmic transformation is indicated when there is a proportional relationship between the standard deviation and the mean when calculated on the arithmetic scale. This relation implies that the groups have constant coefficients of variation, and therefore equal standard deviations when expressed on the logarithmic scale (Aitchison & Brown, 1957). If this is so, the usual analysis of variance procedures can be applied after transformation to the logarithmic scale. Evidence that this proportional relationship holds for the body weights of growing mice is provided by the results published by Chai (1956*a*, *b*). This worker measured 60-day weights of eight groups of mice of comparable genetic variability ranging from 14 to 37 g. in mean values, and found that the arithmetic standard deviations were proportional to the means. Also, Howard (personal communication), who took weekly weights from birth to 10 weeks of age in large samples of inbred and F_1 hybrid mice, found proportionality between standard deviation and mean when comparing the different age groups for the first 4 weeks of life, the period to which our data relate.

We have accordingly transformed all weights into logarithms for the calculation of means and other statistics. The test for outliers described earlier was carried out on the transformed data.

Crude growth curves. Fig. 1 shows the mean body weights in the three environments week by week. The means were calculated on the logarithmic scale, but for graphic presentation have been reconverted into grammes. As explained in the next section, effects of environmental treatments cannot be accurately assessed from crude growth data uncorrected for litter size. On the other hand, the sex difference in growth, which is a constant feature of Fig. 1, is relatively unaffected by litter size. It attains significant proportions in all three environments and is in line with the generally observed fact that male mice grow faster than females (e.g. Falconer, 1953; Chai, 1956*a*). For this reason the two sexes are separately treated in the ensuing analysis.

Relation between body weight and litter size. The more mice there are in a litter, the smaller they are at birth and the slower they grow. In comparing our growth data at different environmental temperatures we must take this effect into account, since early mortality substantially reduced the average litter sizes of the groups reared in the hot and cold environments as compared with those reared in the temperate environment. A weighted linear regression equation of the form:

$$y_x = \bar{y}_w + b\,(x - \bar{x}_w),$$

is fitted to the data for each age group in each environment. y_x is the expected mean log body weight for a litter containing x mice; $\bar{y}_w$ and $\bar{x}_w$ denote weighted means, and the regression coefficient, b, states the amount by which the mean is on average changed by each additional mouse in the litter. In calculating the regression the weighting (w) given to each mean was the number of individuals contributing to it. The methods of calculation are described by Quenouille (1952).

To illustrate, we may take the data on 4-week mice raised in the cold (Table 4).

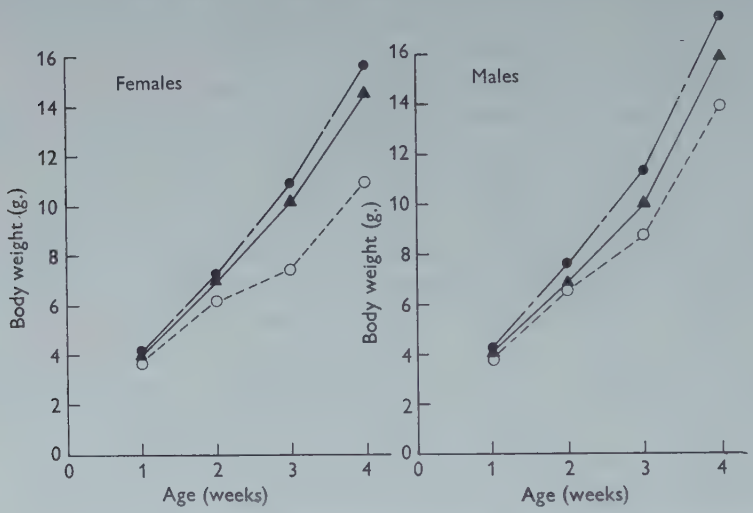

Fig. 1. Mean body weights of female and male mice in the three environments at 1–4 weeks of age. Hot; ●——●; Temperate, ▲—▲; cold, ○- - -○.

Table 4. *Specimen sheet of data required for calculating weighted regression coefficients* (*4-week-old mice reared in the cold environment*)

Litter no.	Litter size (x)	Males		Females	
		No. (w_{σ})	Mean log weight (y_{σ})	No. (w_{φ})	Mean log weight (y_{φ})
1	7	4	1·0593	3	1·0147
3	3	2	1·1050	1	1·0790
4	7	3	1·1883	4	1·1157
5	3	3	1·2053	0	—
7	10	3	0·7847	7	0·7059
8*	7	1	1·0370	5	1·0178
10	7	2	1·2085	5	1·1788
12	5	2	1·2410	3	1·2237
13	2	2	1·1655	0	—
14	4	3	1·2560	1	1·2100
15	4	3	1·2043	1	1·1880
19	3	3	1·1133	0	—
20	6	2	1·1740	4	1·1595
23*	4	3	1·3153	0	—
27	8	2	1·0370	6	1·0523

* When $x > (w_{\sigma} + w_{\varphi})$, runts were present in the litter and have been omitted.

Weighted regressions:

Males: $y_{\sigma} = 1{\cdot}140 - 0{\cdot}039\,(x - 5{\cdot}316)$; $s_b = 0{\cdot}012$, D.F. 13.

Females: $y_{\varphi} = 1{\cdot}038 - 0{\cdot}082\,(x - 7{\cdot}175)$; $s_b = 0{\cdot}018$, D.F. 9.

The fitted regression equations are shown at the foot of the table. Both coefficients are highly significant ($0{\cdot}01 > P > 0{\cdot}001$), indicating a marked dependence of body weight on litter size. They are negative in sign, showing that the larger the number of individuals in the litter the smaller the mean body weight.

Since all weights have been transformed into logarithms, the regression coefficients are themselves logarithms. To interpret on the arithmetic scale we take antilogarithms. Thus, in Table 4 the antilog of the regression coefficient for females is 0·83, and therefore in the cold each additional mouse present in a litter at 4 weeks decreases the expected mean weight in grammes of the females in the litter by 1·00 − 0·83, or 17%. Similarly, in the males an additional mouse in a litter decreases the expected mean weight by about 9%.

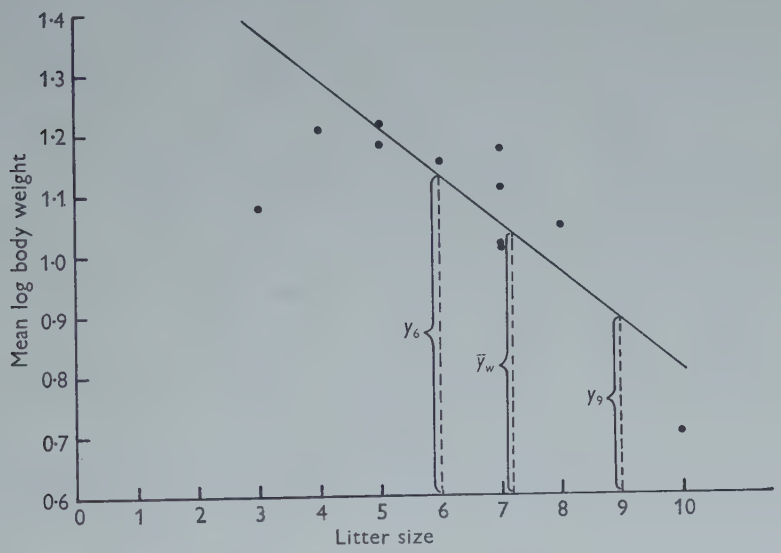

Fig. 2. Regression of mean log body weight upon litter size for 4-week-old female mice in the cold environment. The heights of the lines y_6, $\bar{y}_w$ and y_9 estimate the mean log body weight corresponding to litter size 6, the weighted mean litter size, and litter size 9, respectively.

In Fig. 2 the litter means for females have been plotted against litter sizes and the fitted regression line has been superimposed. There is a suggestion of curvilinearity, and this is also found when the data on the males in Table 4 are similarly plotted. This may reflect the fact that in the cold some of the smallest litters at 4 weeks consisted of the survivors of initially large litters which had suffered catastrophic mortality. Such survivors might be expected to be stunted by the same factors which killed their sibs, and hence to fall below the fitted straight line at the left-hand end. Nevertheless, we have considered the use of linear regressions adequate for the limited purposes in view.

The statistical comparison of the regression coefficients for the data on males and females in Table 4 is difficult because the body weights of the two sexes in a litter

cannot be assumed independent. Thus simple tests of the homogeneity of parameters are not available. The following approximate method has therefore been adopted.

Let m_{σ} and m_{φ} be the mean log body weights of the males and females, respectively, in a litter, and let there be w_{σ} males and w_{φ} females. If there is no difference between the regressions associated with each sex we expect $d = m_{\sigma} - m_{\varphi}$ to be constant for all litter sizes. This may be examined by calculating the weighted linear regression of d on litter size (x), the weighting attached to d being given by $w_{\sigma}.w_{\varphi}/(w_{\sigma}+w_{\varphi})$. This weighting factor gives greatest weight to those values of d calculated from litters with the largest numbers of mice, and from litters where the distribution of males and females tends to equality.

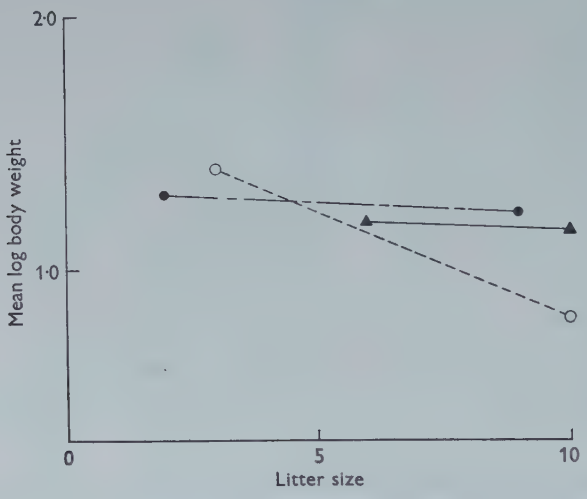

Fig. 3. Regression lines of mean log body weight upon litter size for 4-week-old females in the three environments, for the range of litter sizes encountered in each environment. Hot, ●——●; temperate, ▲—▲; cold, ○----○.

The coefficient of the weighted regression of d on x, calculated from the data of Table 4, is 0·0063, D.F. 9, S.E. 0·0047, and is not significantly different from zero. Hence, in this week and environment the relation between body weight and litter size does not differ significantly between the two sexes.

Comparison of treatments. In this section we discuss the comparison of the effects of the three climatic environments at a given age and sex. To illustrate we may consider the data obtained on the female mice at week 4. The regressions of mean log body weight on litter size have been calculated for each environment, and are shown in Fig. 3. The slopes of these regression lines are heterogeneous ($P < 0{\cdot}001$), indicating that the effect of litter size on body weight differs between environments. A similar result is found with the data on the males at this age. Thus, the result of any comparison of body weights between environments will depend on the value of the litter size at which the comparison is made, the choice of any one litter size being arbitrary. In Fig. 3 the ranges of litter sizes at week 4 for the three environ-

ments is also shown. There are considerable differences due to the differential mortality described earlier. It is seen that the range of litter sizes common to all environments is 6–9 inclusive, a finding constant over all weeks. Thus, we have chosen to present mean log body weights for litter sizes at either end of the common range, i.e. litter sizes 6 and 9.

The biometrical methods illustrated above will now be applied to the assessment of the complete data.

Table 5. *The mean log body weights for litter size* 6 (y_6) *and litter size* 9 (y_9) *and the regression coefficients of mean log body weight on litter-size* (b), *for each environment at each week*

Sex	Week	Environment	y_6	y_9	$b \pm s_b$ (D.F.)
Sexes combined	0	Hot	0·21	0·14	−0·023 ± 0·004 (20)***
		Temperate	0·17	0·10	−0·024 ± 0·002 (16)***
		Cold	0·14	0·11	−0·012 ± 0·003 (18)***
Females	1	Hot	0·62	0·56	−0·019 ± 0·007 (20)*
		Temperate	0·63	0·60	−0·012 ± 0·010 (15)
		Cold	0·62	0·50	−0·041 ± 0·013 (11)**
	2	Hot	0·85	0·72	−0·043 ± 0·010 (17)***
		Temperate	0·88	0·82	−0·020 ± 0·008 (16)*
		Cold	0·86	0·68	−0·059 ± 0·015 (11)**
	3	Hot	1·02	0·92	−0·032 ± 0·010 (17)**
		Temperate	1·04	0·99	−0·018 ± 0·008 (16)*
		Cold	0·97	0·72	−0·083 ± 0·013 (9)***
	4	Hot	1·19	1·12	−0·021 ± 0·010 (17)
		Temperate	1·17	1·15	−0·007 ± 0·008 (16)
		Cold	1·13	0·89	−0·082 ± 0·018 (9)**
Males	1	Hot	0·62	0·55	−0·022 ± 0·007 (20)**
		Temperate	0·61	0·60	−0·005 ± 0·012 (16)
		Cold	0·57	0·52	−0·015 ± 0·010 (14)
	2	Hot	0·86	0·76	−0·034 ± 0·011 (17)**
		Temperate	0·85	0·82	−0·010 ± 0·010 (16)
		Cold	0·81	0·72	−0·030 ± 0·010 (14)**
	3	Hot	1·04	0·95	−0·027 ± 0·010 (17)*
		Temperate	1·00	0·99	−0·004 ± 0·012 (16)
		Cold	0·92	0·81	−0·037 ± 0·011 (13)**
	4	Hot	1·23	1·17	−0·020 ± 0·010 (17)
		Temperate	1·21	1·19	−0·005 ± 0·011 (16)
		Cold	1·11	1·00	−0·039 ± 0·012 (13)**

* $0{\cdot}05 > P > 0{\cdot}01$; ** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.

The complete data

The results can now be tabulated in a more illuminating form than the graphic presentation of crude growth curves. Table 5 gives means corrected to litter sizes 6 and 9 by the use of regression coefficients calculated separately for each group. The coefficients are also given, together with their standard errors and degrees of freedom.

We can thus directly compare different ages, sexes and environments with respect both to mean log weights and to the steepness of the regression of means

upon litter size. Statistical tests of significance can be made between environments within each age group, since the data are independent, but these tests should not be used to compare ages within an environment since the data are serially correlated.

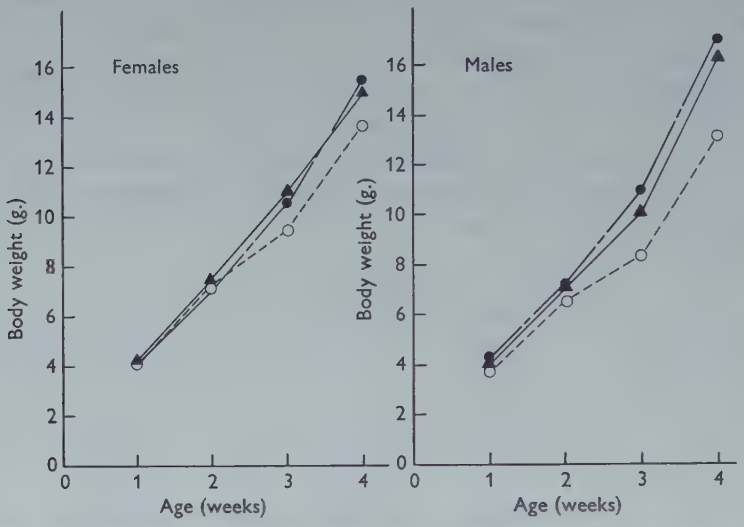

Fig. 4. Mean body weights of female and male mice in the three environments at 1–4 weeks of age, corrected to litter size 6. Temperate, ▲—▲; hot, ●—·—●; cold ○----○.

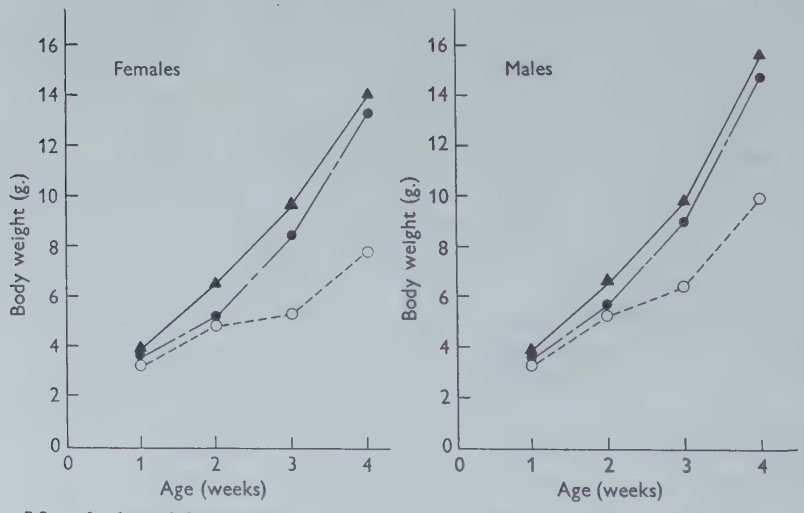

Fig. 5. Mean body weights of female and male mice in the three environments at 1–4 weeks of age, corrected to litter size 9. Temperate, ▲—▲; hot, ●———●; cold, ○----○.

Mean body weights. The week by week means in the three environments, corrected to litter sizes 6 and 9, respectively, are plotted in Figs. 4 and 5. We have again reconverted the means to the arithmetic gramme scale for illustrative purposes. Growth in the cold environment is clearly depressed to a considerable degree,

particularly for large litters, and the effect is significantly greater in females than in males. On the other hand, the difference between the mice reared in the hot and temperate environments is small and not significant.

Barnett & Manly (1956) found that the weight of mice 3 weeks of age was significantly depressed at −3° C. in two out of three inbred strains tested, but not at 10° C.

Comparison of regression coefficients. It seems reasonable to assume that the regression of log body weight on litter size is a measure of the degree of competition between individuals in a litter. Inspection of the values of *b* in Table 5 suggests that in the cold the effect of litter size is much more severe than in the other environments. The regression coefficients calculated from the weights at 4 weeks of both the male and female mice reared in the cold are significantly larger than the corresponding regression coefficients for the other environments. The development of this effect, week by week, is shown in Fig. 6. Although the graphs suggest strongly that the effect is more severe in the females there is no significant sex-difference in the regression coefficients at either week 3 or week 4 in any of the environments.

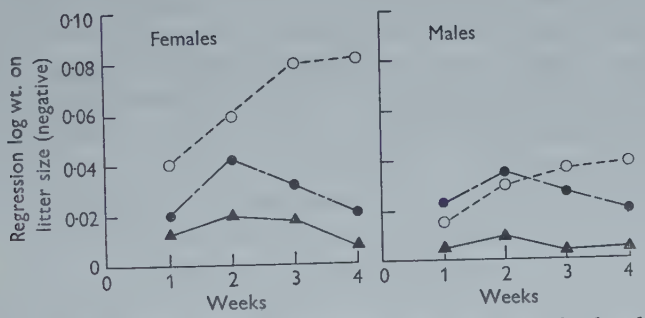

Fig. 6. Regression coefficients of mean log body weight upon litter size, for female and male mice in the three environments at 1–4 weeks of age. Temperate, ▲—▲; hot, ●---●; cold ○----○.

In the case of the mice reared in the hot and temperate environments the litter-size effect reaches a peak at 2 weeks and then subsides. This might be expected since the peak corresponds to the period of greatest competition for the limited maternal food supply. But in the cold environment the effect is still increasing steeply at 4 weeks, suggesting that competition, and hence partial dependence on maternal lactation, is still continuing.

Effect on post-natal development

Two indices of post-natal development, as distinct from growth, were measured: the opening of the eyes at week 2 and the opening of the vagina in females at week 4. The data in Tables 6 and 7 show that by both criteria development was retarded by the cold, but unaffected by the hot, environment. It also appears that within a given environment factors retarding growth also retard development. In Table 8 the number of mice reared in the cold with eyes open at week 2 is tabulated for

Table 6. *The number of mice with open eyes at 2 weeks of age, in each of the three climatic environments*

Environment	No. open	No. shut	Total
Hot	90	0	90
Temperate	118	3	121
Cold	39	45	84

Table 7. *The number of female mice with open vaginas at 4 weeks of age, in each of the three environments*

Environment	Patent	Not patent	Total
Hot	17	29	46
Temperate	16	53	69
Cold	3	39	42

different body-weight groups. The sexes were in agreement and have been combined. There is a clear tendency for the heavier mice to open their eyes earlier than the lighter ones. The females reared in the hot and temperate rooms are treated in the same way in Table 9 with respect to vaginal opening. Again rate of development is correlated with growth.

Table 8. *Number of cold-reared mice with eyes open at 2 weeks of age, arranged according to body weight*

	Body weight (log grammes)											Total
	0·50–0·54	0·55–0·59	0·60–0·64	0·65–0·69	0·70–0·74	0·75–0·79	0·80–0·84	0·85–0·89	0·90–0·94	0·95–0·99	1·00–1·04	
Eyes open	0	0	0	1	0	5	4	9	11	7	2	39
Eyes shut	6	5	5	7	6	3	4	9	0	0	0	45
Total	6	5	5	8	6	8	8	18	11	7	2	84

Table 9. *Number of females reared in the hot and temperate rooms having vaginas patent at 4 weeks of age, arranged according to body weight*

Environment	Vagina	Body weight (log grammes)									Total
		0·95–0·99	1·00–1·04	1·05–1·09	1·10–1·14	1·15–1·19	1·20–1·24	1·25–1·29	1·30–1·34	1·35–1·39	
Hot	Patent	0	0	1	2	3	2	4	4	1	17
	Not patent	0	1	5	4	6	5	8	0	0	29
	Total	0	1	6	6	9	7	12	4	1	46
Temperate	Patent	0	0	0	0	6	6	3	1	0	16
	Not patent	1	1	10	15	17	8	1	0	0	53
	Total	1	1	10	15	23	14	4	1	0	69

SUMMARY

Pregnant mice were placed in rooms at three different environmental temperatures: hot, temperate and cold. The hot and cold environments were less favourable than the temperate as judged by incidence of both prenatal and postnatal mortality of the young. The growth and development of the young was studied during the first 4 weeks of life.

Body weight was expressed on the logarithmic scale throughout, and since males were found to be significantly heavier than females the two sexes were considered separately. The inverse relation between body weight and number of mice in a litter, reflecting competition between litter mates, was particularly marked in the cold and was still increasing 4 weeks after birth. In the hot and temperate environments the effect reached a maximum at 2–3 weeks of age.

When allowance had been made for the effect of litter size on body weight, no significant differences in rate of growth or development were found between the mice in the hot and temperate environments. Both growth and development were markedly retarded in the cold.

We would like to express our thanks to Prof. N. J. Scorgie for the generous provision of facilities in his department, and in particular for the loan of the temperature-controlled rooms; to Prof. Scorgie, to Prof. E. C. Amoroso, F.R.S., and to Dr R. E. Glover, for the encouragement which they gave to this work; and to the Agricultural Research Council for financial support of two of us (A.M. and D.M.).

REFERENCES

Aitchison, J. & Brown, J. A. C. (1957). *The Lognormal Distribution.* Cambridge University Press.
Barnett, S. A. & Manly, B. M. (1956). Reproduction and growth of mice of three strains, after transfer to −3° C. *J. Exp. Biol.* **33**, 325–9.
Chai, C. K. (1956*a*). Analysis of quantitative inheritance of body size in mice. I. Hybridization and maternal influence. *Genetics*, **41**, 157–64.
Chai, C. K. (1956*b*). Analysis of quantitative inheritance of body size in mice. II. Gene action and segregation. *Genetics*, **41**, 165–78.
Dixon, W. J. (1953). Processing data for outliers. *Biometrics*, **9**, 74–89.
Falconer, D. S. (1953). Selection for large and small size in mice. *J. Genet.* **51**, 470–501.
Michie, D. (1955). Towards uniformity in experimental animals. *Lab. Anim. Bur.*, collected papers, **3**, 37–47.
Michie, D., McLaren, A., Ashoub, M. R. & Biggers, J. D. (1958) The effect of the environment on phenotypic variability. In preparation.
Quenouille, M. H. (1952). *Associated Measurements.* London: Butterworth.

ON THE FUNCTION AND EVOLUTION OF ELECTRIC ORGANS IN FISH

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(With Plates 5 and 6)

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I. INTRODUCTION

The inadequacy of functional and evolutionary theories of electric organs in fish has been apparent for a long time. Fish are the only class in the whole animal kingdom known to possess specific electric organs. This fact appears noteworthy because (i) the material from which these organs are derived, muscular tissue, is commonly present in other groups of animals; (ii) in fish these organs must have evolved several times independently. This latter conclusion is based first on the occurrence of electric organs in widely unrelated families, and secondly on the fact that electric organs are developed from different parts of the body.

Although it is frequently stated that electric organs are remarkable adaptations, e.g. in having the electric elements suitably arranged in parallel or in series to conform to the conductivity of either fresh or sea water, very little has been known until recently of the biological significance of these organs—apart from a subjectively experienced defensive action and a surmized offensive role. This is probably the main reason why the problem of their evolutionary history, clearly stated by Darwin (1872), still awaits a satisfactory answer. Dahlgren (1910), after a detailed examination of 'the origin of the electricity tissues in fishes', comes to the conclusion 'that the impulse to evolve this tissue is a real inner stimulus working independently of outer conditions.... The evolution of these structures was most probably not based upon a basis of natural selection.'

It has been customary to distinguish between strong electric organs and pseudo or weak electric organs. Most workers in recent years have been concerned with physiological aspects of electric tissue, and have availed themselves mainly of *Torpedo* and *Electrophorus*, both fish giving off strong electric discharges. It appears that an investigation of the weak electric fishes is more likely to reveal possible evolutionary starting points. Apart from the fact, established towards the end of last century, that the weak electric organs are capable of giving off weak electric discharges (Babuchin, 1877; Sanderson & Gotch, 1888; Fritsch, 1891) very little new information has come to light, and the functional significance and evolutionary history of the weak electric organs have remained obscure. Suggestions, which have been put forward in the past, about the role and mode of action of weak electric organs appear implausible; e.g. that weak electric fish, feeding on insect larvae, worms and crustacea may be able to stun their small prey before swallowing

it; or that weak electric fish were simulating strong electric fish, thereby deriving a certain amount of protection.

As has been reported previously (Lissmann, 1951) a specimen of *Gymnarchus niloticus* aroused interest through its navigational abilities. This fish, which frequently swims backwards, appears to avoid obstacles, to find its way through crevices in rocks, and to locate its prey from a surprising distance. This makes it unlikely that either the poorly developed eyes, or any conventional concepts of perception of water currents or water pressure through the lateral line, could account for such a performance. Structurally the species *G. niloticus* has been known to possess small organs, assumed to be electric (Erdl, 1846), although this had not been generally accepted (Fritsch, 1885). Preliminary experiments indicated that these organs were indeed electric, and they appeared to play an important role in the orientation of *Gymnarchus* and also of two other species of teleost fishes. The theory then suggested for this mechanism implied that fish may be able to detect objects at some distance by appreciating their electrical conductivity which can be expected to differ from that of the surrounding water (Lissmann, 1951). It was, therefore, decided to obtain additional information and to investigate these species in their natural habitat in West Africa as well as in the laboratory.

However, working conditions in the field and scarcity of living material in the laboratory have imposed serious limitations on the nature of experiments which could be undertaken hitherto. It cannot be claimed that the observations reported here have reached any degree of completeness; nevertheless, they clarify the picture to a certain extent, and this investigation is being continued as more material becomes available.

The problems of electric organs, which will be dealt with in this and subsequent papers, are mainly concerned with (i) their possible use as part of an orientating mechanism; (ii) the mode of action of this mechanism; (iii) its biological significance. It is hoped that some lines of evolution of the electric organs may thus be revealed.

In this inquiry it seems important that the electric organs should be considered not just as organs interesting in themselves, but rather as a part of the general bodily organization of the fish, having a role to play in the relation between the fish and its environment.

II. MATERIAL AND PROCEDURE

Most of the material used in the present study was collected in 1951 in the course of an expedition to the Northern Territories of Ghana. The fish were caught in the Black Volta and its tributaries Kamba, Sielo, Poni, etc.; some additional species were obtained from other parts of Africa and from South America.

It was noted that the water of the natural habitat of the fish under discussion was extremely turbid in the dry season (February to May); the particles in suspension appear to be very fine as it takes several days before the water, kept stagnant in an aquarium, becomes reasonably clear. During the rainy season, with increasing soil erosion, turbidity becomes even more marked. Under such conditions the eyes of

the fish would appear of little use and this may be expected to favour the evolution of alternative sensory mechanisms.

The presence of electric fish in any particular stretch of river or in drying-up pools could be detected by a pair of electrodes suspended from a boat or from a pole on the bank. The electrodes, copper wire 1 cm. long and 12 cm. apart, were connected to a battery-driven three-stage amplifier and electric pulses were noted either by the use of headphones, or by visualization on a G.E.C. Miniscope (6 V. d.c. using a vibrator). On many occasions it was found possible to hear the discharges, though more faintly, by connecting the headphones directly to the electrodes without any amplification; headphones of 16 and 2000 Ω proved equally suitable. At a later stage pulses observed in rivers and pools were registered for further examination by means of a magnetic tape recorder.

These observations revealed a number of different types of discharges, best seen in the main rivers, where an absence of crowding makes analysis easier. Rhythmic or regular recurrence of similar spikes was taken to mean a single source of emission. Broadly, the types of discharges can be placed in the following categories:

(1) On five separate occasions (Black Volta, Kamba, Sielo) a very uniform hum of approximately 300 cyc./sec. was heard. Although in one instance these very regular pulses were seen on the oscilloscope, it was at that time not possible to make a recording. The spikes were monophasic; the duration of each spike seemed to extend over a few milliseconds. Thus, they clearly resembled the previously noted discharges of *Gymnarchus niloticus*.

(2) Fairly regularly spaced and constant pulses, varying in frequency between 1 and 6/sec., were heard and recorded on frequent occasions. When the electrodes were left in the same position in the water the size of the spikes remained constant for long periods. It was assumed that this indicated relatively stationary specimens (Pl. 5, 1*a*). Sometimes it also happened that the frequency of such pulses—apparently emitted by single specimens—showed periodic variations in frequency (Pl. 5, 1*b*). Whenever a heavy stone was thrown into the river, or a pole thrust in the general direction of the electrodes, the pulses usually quickened and disappeared for good. However, it was also noted on several occasions that when the bottom of the boat was tapped or thumped the pulses disappeared abruptly for a few seconds to reappear with their previous characteristics. All these pulses were polyphasic and each pulse appeared to be of very brief duration, i.e. less than 1 msec. and, therefore, difficult to record photographically in the field in any detail. These discharges appeared similar in frequency and shape to those observed in *Mormyrops boulengeri* (Lissmann, 1951).

(3) Similar pulses of higher frequency, usually between 20 to 50/sec., were also observed and recorded on many occasions without any deliberate disturbance having been caused. Normally such pulses, which could be attributed to a single source, remained in evidence only for a short period of time, i.e. presumably the fish were swimming past the electrodes (Pl. 5, 1*c*). However, in small drying-up pools, where there was a high concentration of fish, electric signals could be picked up almost continuously (Pl. 5, 2).

(4) Only on one occasion four short, but distinct bursts were picked up and heard in the Black Volta; the estimated frequency was about 120/sec.; the duration of each burst was probably of the order of 1 sec. The frequency was appreciably higher than that of *Gymnotus carapo* which had been examined earlier in the laboratory (Lissmann, 1951) and which has a normal frequency of about 50 pulses/sec.

(5) On a number of different days when the headphones were directly connected to the electrodes, and the latter lowered close to a rock into the Black Volta (near Buga), an extremely powerful and brief discharge, consisting of a train of pulses, could be evoked repeatedly. Since the strength of the discharge appeared of an entirely different magnitude from those discussed above, it was ascribed to *Malapterurus electricus*, the only strong electric fish known to exist in that area.

For further investigation in the laboratory attempts were then made to catch the fish which emitted these discharges. In most localities in the rivers it was possible to use cast nets only; in smaller pools a miniature seine net was used with success, and practically all fish could be taken from such pools. On two occasions I benefited from the visit of the Fisheries Officer who operated a large seine net with local fishermen, and this made it possible to extend the collection.

Fish were kept in large, round native clay pots (80 cm. in diameter); although the water evaporated at a considerable rate and had to be replaced at frequent intervals, it remained much cooler than in other containers and the fish survived well. During catching expeditions kerosine tins were found to be useful and manageable receptacles for transport of the smaller species. For observation and experiments a collapsible aquarium ($66 \times 33 \times 35$ cm.) was constructed of rubber-edged Perspex plates which were clamped into a metal frame; two large accumulator jars could also be used for the smaller species. Aeration was provided by diffusing air from an R.A.F. oxygen cylinder which had to be pumped up twice daily by means of a car tyre pump to a pressure of between 40 and 50 lb. Some of the fish were taken to the Department of Zoology, Achimota, for examination and later brought back to Cambridge, and are still alive at the time of writing.

III. *GYMNARCHUS NILOTICUS*

The pulses mentioned under (1) in the previous section were clearly identical with the signals already described (Lissmann, 1951) which are produced by the species *Gymnarchus niloticus*. The characteristics of these discharges are quite distinct from any produced by other species of fish in that area. Unfortunately, specimens were not as abundant as had been expected from records of catches made by Dr K. R. S. Morris at the same localities in previous years. A number of these fish were observed coming to the surface, often near fallen trees in the rivers, where capture appeared impracticable. In all, nine specimens were obtained; some of these were too large to be kept alive for any length of time. In addition to the first fish, 28 cm. in length, observed for 7 months in Cambridge, two more specimens were eventually brought back for further study: they now measure 52 and 54 cm. respectively.

Gymnarchus is a predator which feeds on other fish, and it can attain a considerable length. Svensson (1933) records a specimen of 160 cm. This species has been noted from various parts of East and West Africa north of the equator. The genus is monospecific and represents one of the two suborders of the Mormyriformes, the other being the Mormyroidei (Berg, 1947). Other authorities include this fish as a separate genus amongst the Mormyridae.

Apart from a similar geographical distribution *Gymnarchus* shares with the Mormyroidei a number of anatomical peculiarities, e.g. a vesicle of the swimbladder is applied to the inner ear, it has enlarged valvulae cerebelli, an opercular velum, a caudal electric organ, etc. On the other hand, *Gymnarchus* differs from the Mormyroidei in many striking features: the elongated body is without tail fin, anal fin or pelvic fins, but the fish possesses a very long dorsal fin; the swimbladder is adapted for atmospheric respiration and the circulatory system is accordingly modified; the electric organs are substantially different from those of the Mormyroidea. For further details of the anatomy and development of *Gymnarchus* reference must be made to the publications of Erdl (1947); Hyrtl (1856); Fritsch (1885); Ridewood (1904); Asheton (1907); Dahlgren (1914); Stendell (1914*a*); Pehrson (1945); Omarkhan (1949).

We owe to Budgett (1901) the first detailed field observations of this fish and also a description of its breeding habits. *Gymnarchus* builds a nest of floating vegetation in the middle of which there is a 'private pond'; into this are laid eggs, measuring 1 cm. in diameter. The parent fish keeps guard near the nest and is, apparently, very aggressive. The material of eggs and embryos collected by Budgett forms the basis of much of the subsequent anatomical and embryological work on this species.

The specimens available for the present study measured between 28 and 85 cm. In the laboratory they were kept in electrically heated aquaria at temperatures between 25–30° C. The largest experimental tank measured $120 \times 75 \times 45$ cm. The food consisted mostly of minnows and gudgeons. *Gymnarchus* displays considerable agility in locating and catching its prey; this often appears to be sucked in by an expansion of the branchial basket and is accompanied by a resounding snap. Larger fish are often bitten in half before being swallowed. *Gymnarchus* shows marked cannibalistic tendencies; individuals have therefore to be kept in separate containers. The fish come periodically to the surface for respiration; as the snout breaks the surface of the water a marked longitudinal depression can be observed to form on the ventral side.

Erdl (1846) and Fritsch (1885) have described the electric organ of adult specimens, Fritsch suggesting that it may have some respiratory significance. Dahlgren (1914) has followed the development of this organ up to the 42-day-old embryo. It consists of four 'tube-like' cylindrical structures ('spindles') on either side, extending from the tip of the pointed tail to varying anterior levels. A cross-section through the tail reveals that it is largely made up of these electric spindles; further forward they are closely applied to the median parts of the body. The dorsal and ventral spindles are considerably shorter than the upper median and lower median spindle. Fritsch has found the last traces of the upper median spindle at the level of the pectoral fins: the ventral spindle terminates at the level of the anus. Counts of electroplates composing these organs have been made by Erdl (1846) and by Fritsch (1885) and are given below

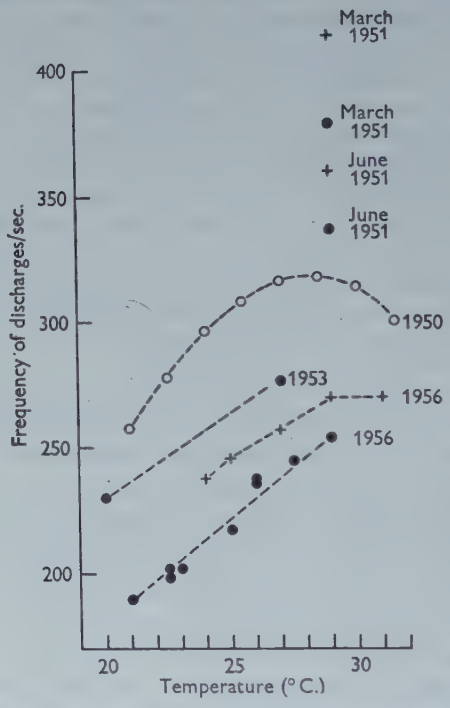
	Erdl	Fritsch
Dorsal spindle	56	99
Upper median spindle	136	139
Lower median spindle	96	111
Ventral spindle	56	99

Along the middle of its course the diameter of a transverse section of a spindle is stated to be 4·2 mm.; this diameter decreases both anteriorly and posteriorly. These figures are for a specimen 89 cm. in length. The maximal length of an electroplate is given as 1·2 cm. According to Dahlgren (1914) the electric tissue of *Gymnarchus* is developed by the differentiation of striated muscle tissue during the embryonic or larval period from the ninth to the forty-second day. Each electroplate is made up of twelve to twenty or more muscle cells, and receives its nerve supply at the posterior face. The nerves take their origin in large cells situated in the spinal cord dorsally to the central canal, and they emerge through the ventral roots forming four longitudinal electric nerves. In captivity these fish frequently lost and regenerated the last 5–7 cm. of the pointed tail. One specimen in particular often regenerated malformations in the shape of hooks or forks; these, however, were again lost after a few weeks and the perfect, pointed tail reappeared. It has never been ascertained how the damage was incurred as there were no sharp objects in the tank. Only on one occasion, when the tail was nearly broken off and trailing, did the animal turn into a circle and viciously attack its own hind end. This strong curvature of the long body axis is not one of the typical movements of *Gymnarchus*, which may seem surprising in a fish of such elongated shape. One of the most immediately striking features of *Gymnarchus* is its locomotory performance: in all phases of the locomotory cycle the body can be kept predominantly straight. As this type of movement has probably some bearing on the electric locating mechanism (see below), it has been examined in some detail and will be reported in a separate paper.

All specimens, when first examined, emitted an uninterrupted sequence of electric pulses at the rate of approximately 300/sec. These pulses can be displayed either with the fish out of water, by placing two electrodes on the surface of the skin, or with the fish in an aquarium by introducing the pair of electrodes into the water. The observations were extended for many hours under varying conditions; neither intense illumination nor complete darkness seemed to alter the frequency of the discharges, nor did the frequency change when a fish, which had been motionless for some time, was suddenly and vigorously stimulated, nor when a starved fish had been given an abundance of food. However, slight but constant differences in frequency were noted between individuals of comparable size under identical conditions. It has not been possible to relate these differences to sex or any other features.

One condition which does affect the frequency in a regular manner is temperature (Text-fig. 1). The temperature of the water observed by Budgett (1901) in the breeding season was 28·5° C., which may perhaps be taken as optimal. Whether diurnal or seasonal variations occur has not been investigated, but a gradual decrease in frequency has been noted in the two specimens which have now been in captivity for over 6 years. In the course of this period the frequency has dropped from about 400/sec. to approximately 280/sec. under comparable temperature conditions (Text-fig. 1). At this stage it is impossible to decide whether this change is related to age, conditions of captivity, or some other factor. No records are available of the largest specimen (85 cm.) as it was too large to be accommodated in an aquarium, but when this fish was examined immediately after capture, lying on dry land, no significant difference was noted in its frequency, compared with that of the smaller specimens. Nor could any shocks be felt by placing wet fingers on its tail and other parts of the body.

As long as *Gymnarchus* is motionless the strength of the individual pulses is remarkably regular and can be easily recorded (Text-fig. 2*b*, *c*). While the fish is swimming or chasing its prey there is no evidence that the voltage alters to any marked extent, but owing to the high attenuation of water and the changing relative position between the fish and the recording electrodes, this is not easily demonstrable with any degree of accuracy. Attempts to determine the voltage of the discharges, with the fish superficially dried and lying on cotton wool, were made on three specimens, by

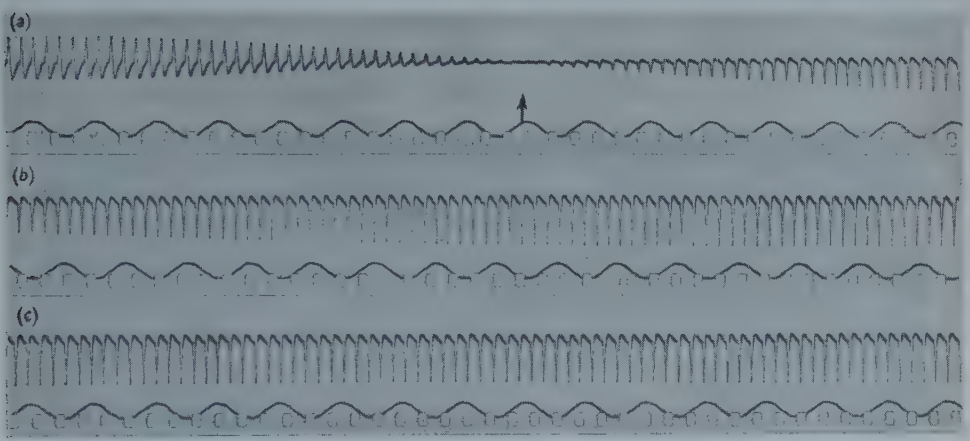


Text-fig. 1. Variations in the frequency of impulses exhibited by three specimens of *Gymnarchus niloticus*. Specimen 1 (○) 28 cm. long; note the change in the frequency in the course of a gradual temperature increase within 10 hr. Specimen 2 (+) and 3 (●); note the decrease in the frequency of discharges from the first record (soon after capture) when the fish measured about 38 cm. up to 5 years later when they measured 52 and 54 cm.

holding one electrode (copper wire) near the tip of the tail and the other about one-third of the body length behind the snout. These fish, measuring 38, 42 and 52 cm., gave each over a period of about 5 min., fairly constant readings of 3, 7 and 4 V., respectively.

That changes in voltage do occur has been noted on the few rare occasions (six in three specimens) when for a brief period the fish failed to emit pulses, after which pulses slowly reappeared with their normal frequency but with gradually increasing intensity. Although this phenomenon of suppression of electric discharges is not reproducible at will, two possible explanations can be offered. In the smallest specimen (28 cm.) it occurred several times when the fish had been

caught after a chase and transported from its aquarium to an experimental tank.* The original explanation that the transport in a small metal container was responsible for the disappearance of the discharges proved fallacious. *Gymnarchus* kept for many days in a kerosene tin continued to discharge, and the discharges even continued in moribund specimens after all motility had ceased. Another possible explanation may be suggested based on two similar observations. The clearest instance occurred in Achimota, when two specimens of *Gymnarchus*, kept in



Text-fig. 2. Continuous record of the electric discharges of *Gymnarchus niloticus* at 23° C. The electrodes, first held close to the posterior end of fish and parallel to its long axis, were rotated by 180° (*a*) and then held stationary (*b*) and (*c*). At ↑ they were pointing to the posterior margin of the dorsal fin. During the discharge the tail becomes negative relative to the more anterior regions of the body. Time marker 50 cyc./sec.

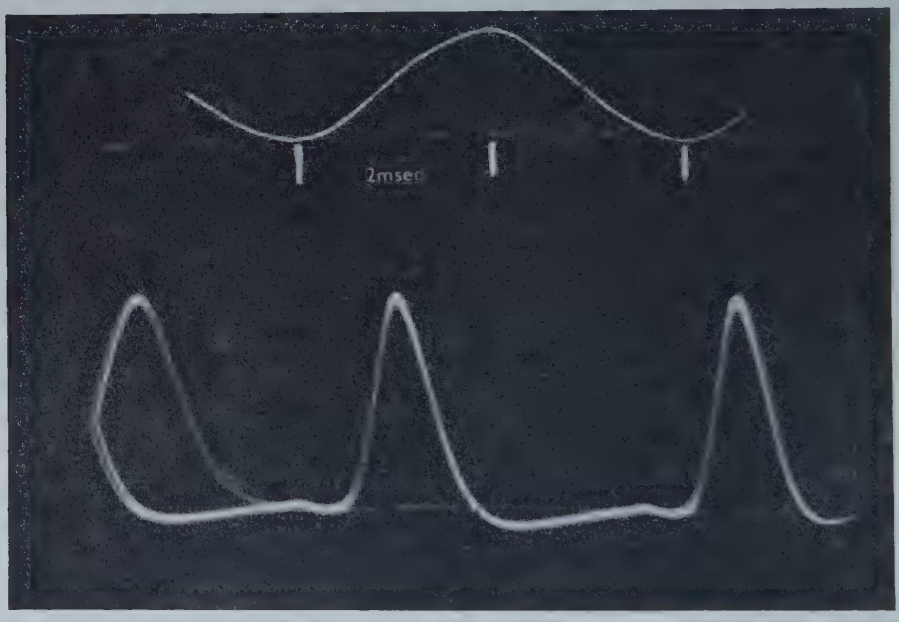
separate tanks, had been given an ample supply of food in the course of an afternoon. At 11.30 p.m. the amplifiers were switched on with a minimum amount of noise and in almost complete darkness. One of the specimens appeared normal in the emission of its electrical discharges, while the other over a period of 5 min. did not show any signs of electrical activity. A dim light revealed that the animal was resting on the bottom, but when touched with a glass rod it began to swim still without emitting any electric pulses. The lights were then switched on and both fish appeared to swim about normally without any change in the electrical behaviour. After 1 min. the non-emitting fish was vigorously prodded with a glass rod; 30 sec. later very faint pulses of approximately normal frequency appeared. They gradually increased, until after about 2 min. the signal strength of both fish was approximately equal. It may be implied that the condition of such specimens may be akin to a state of sleep, although this does not seem to occur very often and only one further similar instance has been recorded despite extended efforts.

When the recording electrodes are placed in the water so that their distance apart is greater than the total length of a *Gymnarchus*, it will be seen that the train of

* Analogous behaviour was shown by small specimens of *Gymnotus carapo* during class experiments; they ceased to discharge after inexpert handling.

discharges emitted by these fish consists of individual pulses 1·3 msec. in duration, followed by an interval of about 2·3 msec. (Text-fig. 3). During each discharge the tail becomes negative relative to the more anterior regions. This is in agreement with the Pacinian rule, since Dahlgren (1914) has shown that the innervated face of each electroplate is situated posteriorly.

The shape of the electric field, set up around the fish during each discharge, has been examined by placing a specimen in a large shallow porcelain tank and introducing two recording electrodes (8 cm. apart) which were mounted equidistant from the centre of rotation of a disk. A long pointer attached to the disk indicated the orientation of the electrodes. The alignment of the electrodes on lines of equipotential (zero reading) and lines of force (maximum) was recorded photo-



Text-fig. 3. Individual pulses of *Gymnarchus niloticus* 27 cm. long recorded at 28° C. The duration of the individual pulse is about 1·3 msec., followed after about 2·3 msec. by the next discharge. Time marker 2 msec.

graphically, together with the position of the fish in the tank. This showed that the line of equipotential normal to the long axis of the fish lies near the posterior level of the dorsal fin (Text-fig. 2*a*). Around this transverse axis, however, the field is not symmetrical, i.e. it is not strictly that of a dipole. This can be demonstrated most easily by placing two electrodes close together (2 cm.) and in line with the long axis of the fish. Movement of the electrodes parallel to this axis will show a maximum at the posterior level of the dorsal fin. Displacement of the electrodes from this position in a posterior direction shows a considerably more rapid decrease of the spikes per distance unit of movement, as compared with anterior displacement. This agrees with the anatomical finding, namely, that whereas all electric spindles terminate at the tip of the tail, they extend to varying anterior levels.

The suggestion that *Gymnarchus* uses the electric discharges as part of a locating mechanism seemed to be supported by a number of preliminary observations, which indicate that the fish is indeed very sensitive to any influences which may affect the electric field set up with each discharge.

(1) The tips of a wire loop, interrupted by a switch, were dipped some 2 mm. into the aquarium water. Normally, unless the fish came very close to the wires, it took no notice. However, when it swam past the wires and the switch was suddenly closed, the fish performed a violent jerk. While this may suggest that the normal configuration of the electric field was upset in this experiment through the outside circuit, it is realized that metals in contact with water will generate on their surfaces electric currents to which a fish may respond (Parker & van Heusen, 1917). Until more quantitative information about the sensitivity of *Gymnarchus* is available, it seems premature to draw any final conclusions from this observation.

(2) It is very obvious that *Gymnarchus* will respond to the presence of metals in the water, although if the metal is left long enough in an aquarium the fish seem to get habituated and shows no behavioural responses. One striking type of behaviour was shown by a small specimen when four straight pieces of bare copper wire, about 50 cm. long, were placed on the bottom of a shallow tank to form a rectangle. When the fish was placed inside this rectangle it appeared trapped within it. Every time it approached a wire, it shied and reversed. When the fish was eventually chased, it slipped over the wire as near as possible to the surface of the water and lying on its side.

(3) The cannibalistic tendencies of *Gymnarchus* have already been mentioned. It appears that two specimens can detect each other's presence at some considerable distance. In order to examine whether or not the emission and perception of electric pulses plays an important role in this, a large shallow tank was filled with water to a depth of about 12 cm. In the middle of this tank a pair of receiving electrodes was fixed to pick up the impulses emitted by the fish. These impulses were then amplified to about 3 V. and continuously adjusted to approximately that value as the fish was swimming about. Round the edges of the tank six pairs of electrodes were immersed and could be successively connected to the output of the amplifier. The fish quite clearly located and attacked whichever pair of electrodes emitted the signals. Within a wide range the frequency of the signals does not appear essential, since the fish behaved similarly to signals of frequencies other than its own, which were fed into the tank by means of a beat-frequency oscillator. This experiment may indicate the general anti-social tendencies in *Gymnarchus*, but it may be expected that under certain circumstances, e.g. in the breeding season, these tendencies are not indiscriminate. No information is available on this point. A study of electrical and other behaviour responses of a breeding pair may be most rewarding.

(4) It was clearly of interest to obtain an indication of the fishes' sensitivity to electrical changes—which must be considerable if the suggested hypothesis is to work—without any direct contact between metal and the water of the aquarium. Two examples may be selected to illustrate the degree of sensitivity of *Gymnarchus*.

(i) The fish shows marked responses to the movement of a rod magnet of a dipole moment of about 5×10^5 e.m.u. operated at some distance behind a screen. At shorter range, *Gymnarchus* can be pulled along, as it were, and reversed by a magnet which is slowly displaced along the slate wall of its aquarium. The fish has also been trained to avoid its feeding place and food when a stationary magnet was placed near it (not visible), and to accept the food when the magnet was removed, and vice versa. Comparable experiments with minnows (*Phoxinus phoxinus*) have, so far, produced no positive results, although these fish could be trained to optical stimuli within 2 days.

(ii) Similarly, *Gymnarchus* has been found to respond to the movement of a small electrostatic charge, such as is produced by combing one's hair with a vulcanite comb.

A more quantitative treatment of this aspect, combined with conditioned reflex experiments will be given in another paper (Lissmann & Machin, 1958).

IV. MORMYRIDAE

Most, if not all the electric discharges mentioned in § II—apart from those of *Gymnarchus* and *Malapterurus*—could be traced to representatives of the family Mormyridae. These are highly aberrant, mostly curious-looking fishes, very variable in the form of body, head and fins (see Text-fig. 5). Boulenger (1909) places them close to the Albulidae or Elopidae and lists ten genera. One or more species of seven of these genera have been examined in the course of the present investigation. These genera include *Mormyrops*, *Petrocephalus*, *Isichthys*, *Marcusenius*, *Mormyrus*, *Gnathonemus* and *Hyperopisus*. Not all the species have yet been identified with any degree of certainty, as the present survey of the electrical behaviour of this family was undertaken mainly in the hope of finding suitable experimental material.

Despite their great diversity in size, form and life habits (Schlesinger, 1909), which will not be discussed here, all representatives share some outstanding structural features. The most noteworthy of these, which probably have some direct bearing on this enquiry are:

(1) The caudal electric organ, first described by Rüppel (1832) as 'two elongated jelly-like masses situated below the tendons of the double-bellied muscles which move the tail fin in a horizontal direction'. In the course of the last century much anatomical and histological work has been done on these organs by Marcusen (1864); Babuchin (1872); Fritsch (1891); Ogneff (1898); Schlichter (1906).* The organ generally extends from the posterior level of the dorsal fin to the tail fin. On either side it is subdivided into a dorsal and a ventral portion, and each of these is composed of 150 to 200 longitudinally arranged electroplates. The nerve supply to this organ emerges from the ventral roots of the spinal nerves in the area of the caudal peduncle. The nerve divides to form a dorsal and a ventral branch of the electric nerve; moreover, there is an interchange of fibres (chiasma) of these peripheral nerves above and below the vertebral column. Babuchin (1877) and Fritsch (1891) have made use of an anuran nerve-muscle preparation to display the

* Szabo (1957).

discharges of Mormyridae, by placing the nerve on the electric organ of a fish. Babuchin observed hopping movements of a toad's leg up to 5 min., giving four to five twitches per second. While these observations can be confirmed, the limitations of the method became obvious after it was established (Lissmann, 1951) that *Mormyrops boulengeri* could give off discharges of considerably higher frequencies. No modern physiological data on the electric organ of Mormyridae appear to be available.

(2) A pair of styliform, longitudinal bones ('Gemminger's bones') are found above and below the electric organs. No comparable structure is known to occur in any other group of fish. Apart from the statement by Marcusen (1864) that these bones have nothing to do with the pseudo-electric organs, and serve as attachment for some tail muscles, little attention has been paid to this peculiarity.

(3) The Mormyridae possess a thick, multi-layered epidermis which extends over the eyes, opercular bones, etc., and it contains small foramina (Marcusen, 1864; Franz, 1921). The basal layer of the epidermis is composed of cylindrical cells; this is followed by three to four layers of polygonal cells. Over this are found regular, more or less hexagonal pillars composed of approximately forty flattened cells. The outermost layer is again formed by three to four rows of polygonal cells. This, as far as is known, is a unique epidermal organization amongst fishes.

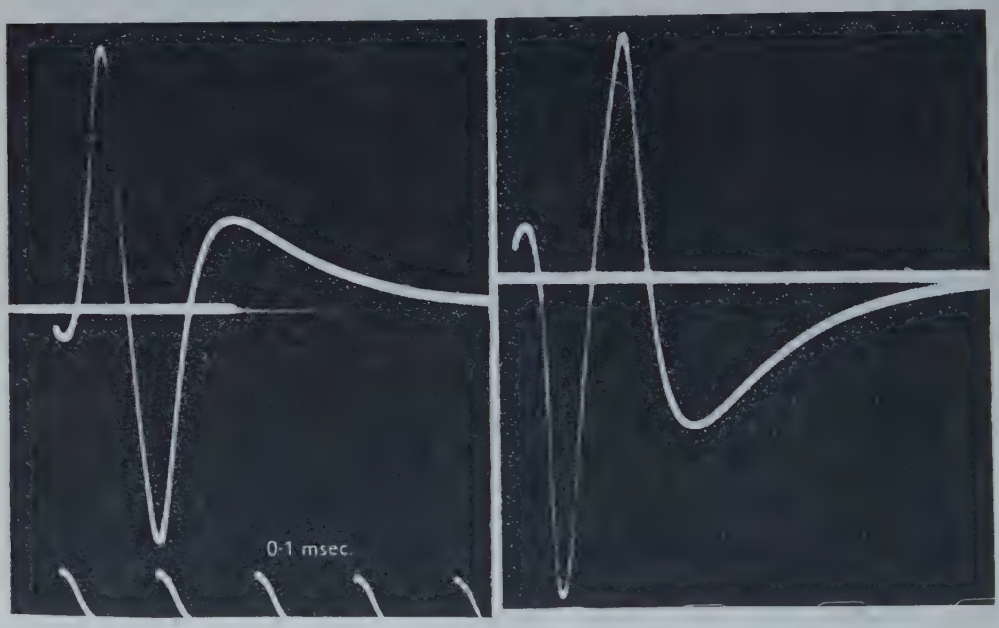
(4) The foramina in the epidermis lead through canals to peculiar cutaneous sense organs which have been a point of some controversy (Franz, 1912, 1921; Stendell, 1914*a*, *b*, 1916). The balance of opinion has now definitely shifted in Stendell's favour and these 'mormyromasts' are considered as specializations of lateralis sense organs (Cordier, 1938; Gérard, 1940).

(5) Similarly, the enormous cerebellum ('mormyro-cerebellum') is now considered predominantly as an association centre for this undefined sense (Sanders, 1882; Franz, 1912, 1921; Stendell, 1914*a*, *b*, 1916; Berkelbach van der Sprenkel, 1915; Suzuki, 1932).

The possible relationships of these structures will be discussed later. Here it may be noted that although the Mormyridae are considered to be related to *Gymnarchus*, their electrical discharge characteristics differ substantially from those of that species. The most striking of these differences are the great variation in frequency which any one individual can produce, and the very short duration of each polyphasic pulse (Pl. 6). Although there are some differences in the electrical behaviour of different species of Mormyridae, the similarities of all the examined species are on the whole greater than the differences. While the basic discharge rate of a resting animal is usually somewhere between 1 and 6 pulses/sec., in extreme cases the pulses can either cease altogether for several seconds or minutes, or conversely they can be accelerated up to about 130 discharges/sec.

The conclusions drawn from observations of electric pulses in the rivers, namely, that resting fish discharge at a lower frequency while in active fish or in disturbed fish (which need not perform any movements) the rate of discharges is speeded up, has on the whole been confirmed by examination of fish in aquaria. A representative selection of records is given in Plate 6. These were taken with the various

species of fish originally at rest (top record) and then excited either by a tap on the aquarium wall or by touching them with a glass rod (lower record). It must be mentioned, however, that bottom-living forms such as *Isichthys henryi* or *Mormyrops breviceps* were more often found resting motionless on the bottom, as compared with some of the smaller, active mid-water species such as *Petrocephalus* and *Marcusenius*. In consequence, it appears that the discharge rate of the latter, even when not disturbed, was rather higher; but after a disturbance their accelerated pulse rate did not persist so long.



Text-fig. 4. Single pulses from the whole organ of *Petrocephalus* sp. (Sudan) 5 cm. long. Distance of recording electrodes 12 cm.; their position is reversed by 180° in the two records. Time marker 0·1 msec.

Two types of stimuli were encountered: some which accelerated and others that slowed down or completely suppressed the discharges. For example, two specimens of *Mormyrus rume* which discharged in regular fashion inside the laboratory ceased to discharge altogether as soon as their aquarium was placed in bright sunlight. The possibility was therefore considered that these discharges might be of special significance when the eyes were unable to function. However, although it was found that switching off the lights in a darkroom produced an accelerated burst of discharges (*Isichthys henryi*, *Mormyrops breviceps*, *Gnathonemus senegalensis*) which often persisted since the animals became more active, a similar burst was elicited when the lights were again switched on.

A considerable difference in behaviour was also noted between animals which had been kept for a long time and had settled down in aquaria and those that had been recently captured and had spent only a few weeks or months in an aquarium.

Thus, in Africa frequencies above 50 pulses/sec. were rarely recorded from fish in captivity, while the highest frequency observed in a river was at least twice that figure. The reason why the search for a mormyrid of such high discharge frequency was fruitless can probably be explained, because a *Gnathonemus senegalensis* which had been kept in an aquarium for over 6 years (before it perished in an accident) did discharge regularly with frequencies up to 130/sec. when touched with a glass rod (Pl. 6). But this same animal—and others—always ceased to discharge when a person approached the aquarium and leaned over it. These silent periods could last up to 30 sec., occasionally punctuated by a single pulse. Eventually the normal discharges were resumed. Analogous behaviour was also noted in other species, particularly in forms with well-developed eyes. A loud noise would sometimes have the same effect.

Thus, most of the observations made in the natural habitat could be repeated on fish in captivity. The rarely noted grouping of discharges into two pulses followed by a longer interval has also been recorded in a few cases from fish in captivity, notably in some specimens of *Hyperopisus bebe* (Pl. 6), but no explanation can be offered for this type of behaviour.

The measurement of the voltage of these discharges was carried out as in the case of *Gymnarchus*, by placing two electrodes superficially on the fish out of water at the levels of the two ends of the electric organ. The results for *Gnathonemus senegalensis*, *Mormyrus rume*, and *Hyperopisus bebe*, 12–19 cm. long, were somewhat variable. 9 to 17 V. were thus registered, but the differences of individual measurements could not be directly related to size or species of the fish.

When a pair of electrodes is placed in an aquarium with an actively swimming mormyrid it will be seen (i) that the size and shape of the pulses displayed on an oscilloscope vary with the relative position of fish and electrodes; (ii) that when the distance between the electrodes is greater than the total length of the fish the individual pulses are polyphasic and, considering that they are emitted from the whole organ, of extremely short duration (about 0·2 msec. in the case of *Petrocephalus*, Text-fig. 4). This seems to indicate a propagated phenomenon over the electric organ but the details of the discharge mechanism are not understood.

While records from rivers and pools frequently indicated solitary specimens, other records showed aggregations or schools of fish (Pl. 5). This seemed to be in contrast to the behaviour of most species in captivity. Despite their small mouths they appeared to attack each other and the damage inflicted led to many casualties. It was noted that the attacks were often directed towards the tail end. As in the case of *Gymnarchus* it can be assumed that this unsociable attitude shows variations throughout the year.

In order to determine whether, in addition to other functions, the electrical discharges may be of social significance some preliminary experiments were carried out in Africa. A cloth partition was fixed in an aquarium dividing it into two equal halves. This partition consisted of a wooden frame over which the cloth was stretched on both sides, so that the two layers of cloth were about 2 cm apart. This frame was fitted into the aquarium by means of plasticine, and could be

expected to be transparent to electrical but not to visual stimuli. One *Gnathonemus senegalensis* was introduced into one compartment of this tank and allowed to settle down for 2 days. After this period a second fish of the same species was carefully introduced into the second compartment. Both fish were resting motionless on the bottom. Recording electrodes introduced into the tank showed that both fish were discharging at a fairly low and regular rate. Whenever one of the two specimens was gently touched with a glass rod its discharge rate went up abruptly and the fish in the other compartment usually followed suit. When one of the fish was removed, a similar movement of the glass rod in the empty compartment remained without effect. These two specimens were left in the tank overnight. After darkness the light of a dim torch showed them both swimming up and down on the opposite sides of the partition, obviously taking note of each other's presence and discharging with higher frequencies. Bursts of discharges from both fish coincided when they came close together, but no correlation in the timing of individual pulses was noted. This observation, though not conclusive, does suggest that the electrical discharges may play a social role in the life of the Mormyridae.*

On the other hand, I have completely failed to note any effect of the discharges on the behaviour of the prey of the Mormyridae; *Daphnia*, *Chironomus* larvae, *Tubifex* and *Enchytraeus* appeared equally unaffected.

V. GYMNOTIDAE

The family Gymnotidae is considered to be an offshoot of the Characidae (Boulenger, 1904). Thus, according to the generally accepted characters, they are not assumed to be of close relationship to the Mormyridae. Although some anatomical and histological information about the Gymnotidae is available, it is largely restricted to *Electrophorus electricus*, and is more scanty than comparable information of the Mormyridae. Nevertheless, a great many structural and functional similarities are found in both these families (Text-fig. 5). Of particular interest is the mode of locomotion which is closely analogous to that of *Gymnarchus*, except that in this instance the long anal fin acts as propulsive organ.

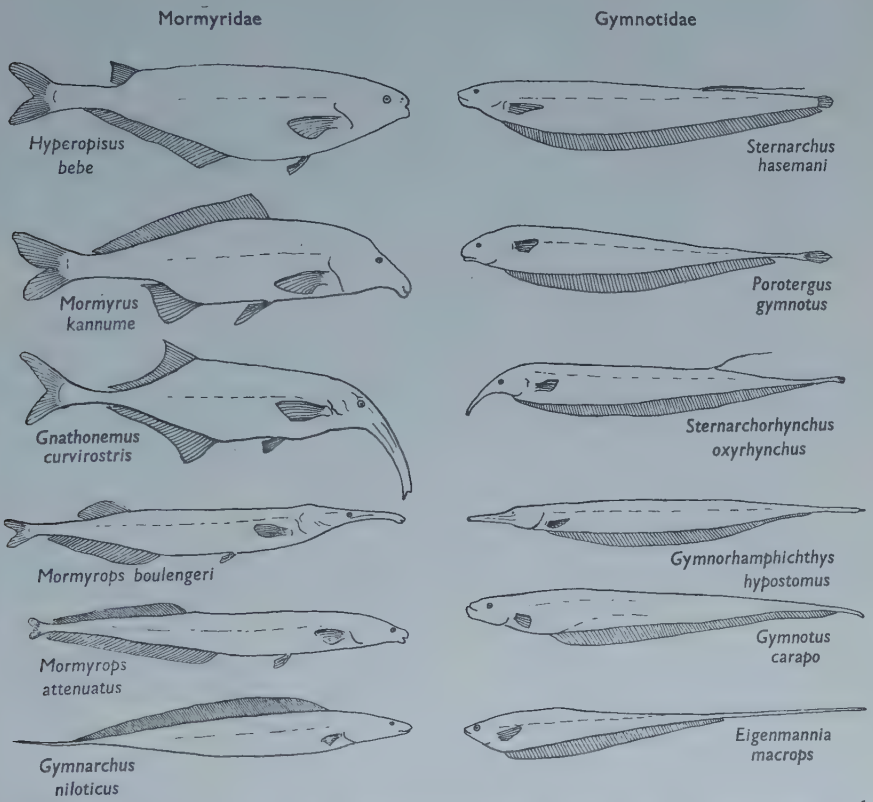
Systematists have separated *Electrophorus electricus* from the rest of this group as being an 'electric fish' (Regan, 1911; Ellis, 1913), although structurally similar electric organs have been described in other genera or subfamilies (Ellis, 1913; Lowrey, 1914; Schaffer, 1917). Since the fact that *Gymnotus carapo* gives off weak electric discharges was reported (Lissmann, 1951) a number of other gymnotids have been described as electrogenic (Coates, 1955; Couceiro, Leao & Castro, 1955; Grundfest, 1957), and it may reasonably be assumed that, like the Mormyridae, all Gymnotidae give off electric discharges.

Compared with the Mormyridae, the family Gymnotidae comprises fewer species. Nevertheless, taken as a group they show a wider range of discharge patterns and frequencies, and between 2 and 1000 impulses/sec. have been reported (Grundfest, 1957).

In the course of the present study four species have been examined: *Gymnotus*

* In this respect there appears to be substantial agreement with a recent note on the subjcet by Moehres (1957).

carapo, *Eigenmannia virescens*, *Hypopomus brevirostris*, and *Staetogenes elegans*. Examples of the discharges of these fish are given in Text-fig. 6. It will be seen that they are of two types: those of *Eigenmannia* and *Hypopomus* which, apart from frequency differences, resemble the discharges of *Gymnarchus*, and those of *Gymnotus carapo* and *Staetogenes elegans*. The latter are brief and diphasic or polyphasic and, therefore, more like mormyrid discharges, although the basic discharge

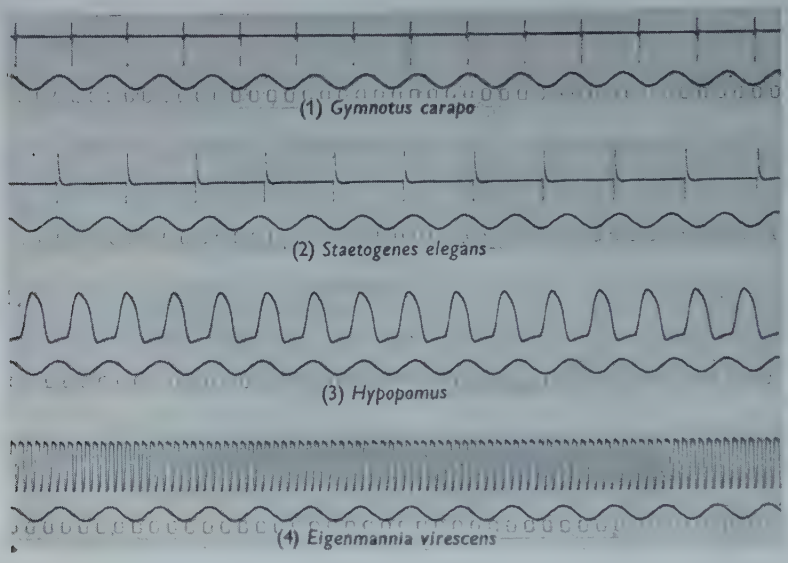


Text-fig. 5. Representative types of the Mormyridae and the Gymnotidae. The convergent evolution between these unrelated families finds its expression in the electric discharges, the reduction of the tail fin, propulsion through an elongated unpaired fin, development of long snouts and several other features.

frequency is considerably higher and does not show so much variation with the state of excitation. However, I find the variation to be considerably greater than the 10 to 20% reported by Coates, Altamarino & Grundfest (1954) as can be seen from Text-fig. 7; but the increase in frequency is not as persistent as in many Mormyridae.

Different individuals of the species examined here show a considerable amount of variation in the frequency of their discharges. Twenty specimens of *Eigenmannia* (10–19 cm. in length) have been recorded; it has not been possible to relate this difference in discharge frequency to differences in size or to any other condition. A specimen of *Hypopomus* 27 cm. long recorded at 25° C. showed a frequency of

about 60 pulses/sec., while a specimen of 23 cm. and at the same temperature discharged at the rate of about 90/sec. These differences have persisted over a period of weeks without much change. As in *Gymnarchus*, temperature is of direct influence on the discharge rate (Text-fig. 8).

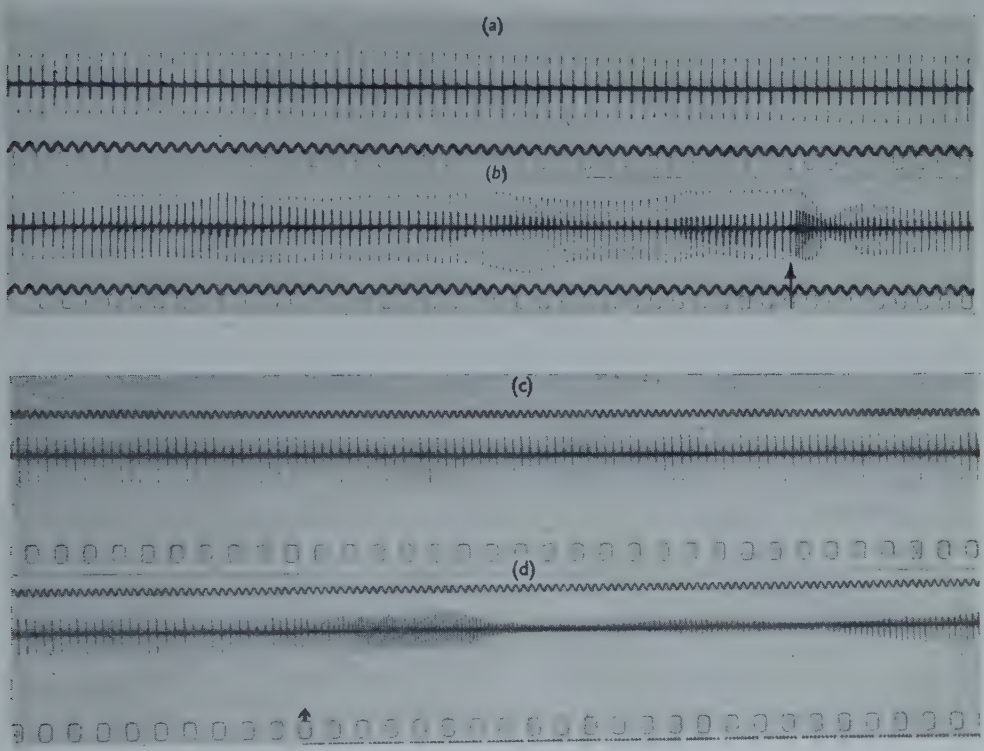


Text-fig. 6. Discharges of four species of Gymnotidae at 25° C. (1) *Gymnotus carapo*, 15 cm. long. (2) *Staetogenes elegans*, 9 cm. long. (3) *Hypopomus brevirostis*, 27 cm. long. (4) *Eigenmannia virescens*, 15 cm. long. Note the short duration and diphasic nature of the pulses in the two upper records. Time marker 50 cyc./sec.

Staetogenes elegans has been of particular interest, because Ellis (1913) and Lowrey (1914) have reported that the submental filaments of this species are structurally identical with the tissue of an electric organ. However, this shy, nocturnal fish is not easily subjected to experimentation. In a normally illuminated room it seems to spend most of the time lying on its side on the bottom of its aquarium emitting regular diphasic pulses (Text-fig. 6), similar to those of *Gymnotus carapo*. No subsidiary activity has, so far, been noted from the head region under such conditions. In a dark room the fish swims actively in mid-water and feeds; as soon as the lights are switched on it rolls over on its side and sinks to the bottom, possibly simulating a dead leaf.

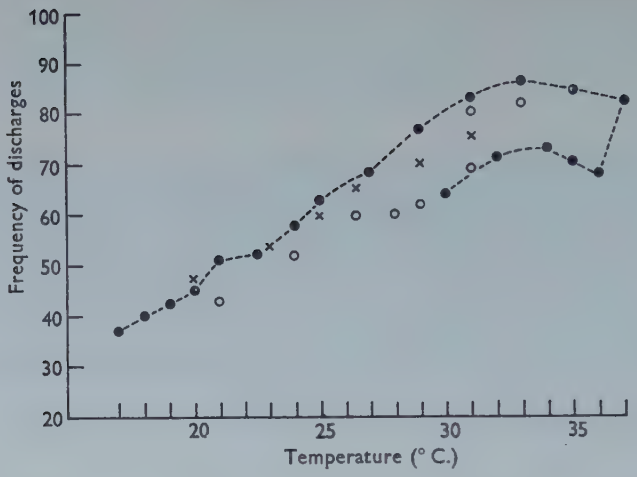
A number of attempts have been made to plot the electric field which is set up with each discharge by *G. carapo*. The fish was placed in a fold of a nylon stocking which was kept taut by two thin Perspex rods clamped the distance of the length of a fish apart and dipped in near the surface of the water in a large shallow tank. Although this prevented the movement of the fish to some extent, after a while most specimens began to struggle and a complete plot for any one specimen was not achieved. By placing one electrode successively along various levels of the fish,

and fitting a second electrode to a pantograph and drawing for each position the lines of equipotential a picture of the field could be obtained. As the example in Text-fig. 9 shows, the line of equipotential which is normal to the long axis of the body lies further foward in *Gymnotus* as compared with *Gymnarchus*.

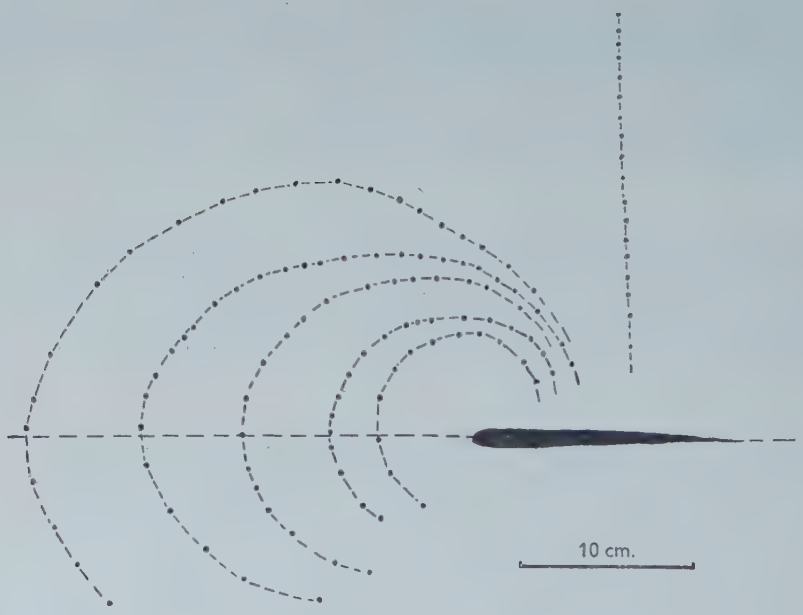


Text-fig. 7. Discharge frequency of *Gymnotus carapo* before and after excitation. (*a*) A fish 17 cm. long at 25° C. discharging at the rate of 60 pulses/sec. (*b*) After excitation the discharge rate goes up to 100 pulses/sec., and for brief periods after being touched with a glass rod (↑) up to 200 pulses/sec. (Continuous record.) (*c*) Another fish, 13 cm. long at 20·5° C. discharging at the rate of 43 pulses/sec. when at rest. (*d*) The same fish after a piece of earthworm has been dropped in front of it (↑). During the next $\frac{1}{8}$ sec. the discharge rate goes up to 100 pulses/sec. Time marker 50 cyc./sec.

G. carapo is a hardy fish which depends partly on atmospheric respiration and is a voracious feeder. Preliminary experiments were, therefore, undertaken to test its suitability for conditioned reflex experiments. This fish shows similar sensitivity towards stimuli induced by a magnet, an electrostatic charge or the introduction of a conductor into the water of its aquarium as have been described for *Gymnarchus*, but unlike *Gymnarchus* it seems to rely to a greater extent on its eyes. Training of this fish was therefore undertaken in a semi-dark room, and the critical tests, during which the reactions of the fish were automatically recorded, were carried out in complete darkness. The arrangement is illustrated in Text-fig. 10*a*. An all-glass



Text-fig. 8. The effect of temperature on the discharge frequency of *Gymnotus carapo* (three specimens). (1) A fish of 22 cm. long (○) increasing its frequency as the temperature is increased. (2) A fish of 15 cm. (×) decreasing the frequency with decrease of temperature. (3) A fish of 13 cm. (●) responding with increased frequency to an increase of temperature from 17 to 33° C. Further warming to 37° C. led to a slight drop. Subsequent cooling produced lower values as compared with previous readings at the same temperature. The temperature was changed in all instances at the rate of 1° C. per 15 min.



Text-fig. 9. Plot of the electric field round the head of a *Gymnotus carapo*. The lines of equipotential are indicated.

aquarium was divided by a partition into two halves. Before the trial the fish was placed in one half while a small piece of earthworm on a thread was introduced into the other half. The thread was stretched between a Perspex rod which dipped into the water and a recording lever writing on a smoked drum. When the partition was removed, which was synchronized with the start of the recording drum, an unconditioned fish would after a while find and accept the food, and the pull on the thread was registered on the drum. The training was of the reward-punishment type. It was found, however, that the method frequently used as punishment in similar fish-training experiments, namely, a knock with a glass rod on the snout, had no effect in the case of *Gymnotus carapo*. Even after quite hard knocks the fish attacked the glass rod and persisted with these attacks continuously for up to 10 min. When after several days the fish had not learned to respect the rod, this was replaced by a T-shaped 'punishment' device. The vertical arm of the T was a handle of Perspex, and the cross-bar a thin piece of aluminium 10 cm. long. At first it was sufficient to dip the metal just below the surface of the water above the fish to chase it away. In prolonged trials the original effectiveness of this method faded to a certain extent, but even in hardened animals a slight touch was adequate as the maximal punishment.

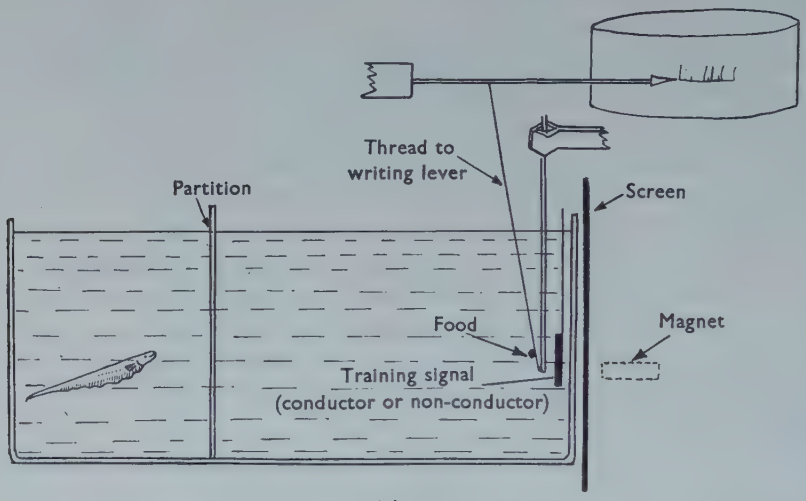
A well-trained fish usually took the food within 4 to 5 sec. on presentation of a positive stimulus, and if it did not approach the food within 30 sec. (initially 60 sec.) during a negative stimulus, this was counted as the correct response. When small pieces of food were used as reward up to sixty trials could be carried out each day; some fish had learned their task within 2 days and showed little or no improvement after that time.

The discrimination of conductors from non-conductors was tested first. Several fish, in separate experiments, were made to take the food when it was presented in front of a Perspex disk 5 cm. in diameter, and not to take it in the presence of a 5 cm. aluminium disk. Having learned this task under ordinary light conditions the fish were presented with the choice between the aluminium disk and a Perspex disk painted with aluminium paint (insulator). There was no hesitation and the insulator and conductor were distinguished as before. Since a number of different disks of both types were used, and since both types were practically indistinguishable to our eye, this seemed to indicate that the conditioning stimulus was not optical in nature.

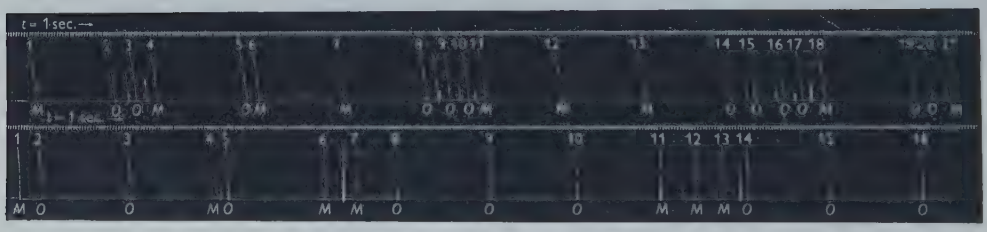
The experiments were then continued in complete darkness. The Perspex disk could be substituted by a glass disk or a Tufnol disk, and the aluminium disk by any metalic disk without affecting the reactions. One may, therefore, conclude that chemical stimuli played no role in this training and that the operative stimulus was electrical in nature.

The converse experiment, using the conductor as the positive stimulus and the non-conductor as the negative stimulus, was carried out on two other specimens of *G. carapo*. Initially the behaviour of the fish towards this 'positive' stimulus was different: it approached the metallic disk slowly and in a fighting posture (strong lateral curvature and periodic thrashing of the body). The disk was frequently

attacked and bitten before the worm was accepted. But the use of hungry fish and the placing of the disk in the early trials at some distance from the food soon resulted in good performance and learning. Paradoxically, at some intermediate stage the attacks were transferred to the Perspex disk.



(*a*)



(*b*)

Text-fig. 10. (*a*) Arrangement for training experiments to conductors and non-conductors and to a stationary magnet. (*b*) Record of two specimens of *Gymnotus carapo* (I and II) conditioned to a stationary magnet. The numbers (1–20) upper record and (1–16) lower record indicate the beginning of each trial when a partition was removed in the aquarium giving the fish access to the food; simultaneously the recording drum was started. The food was presented on a thread, which was attached to a lever recording on a smoked drum the pull exerted by the fish when the food was accepted. *M* denoted the presence, *O* the absence of a magnet behind a screen near the food; the sequence was determined by the toss of a coin and is identical in both records. Fish I (upper record) had been conditioned to take the food in the absence of the magnet, and not to accept it within 30 sec. in the presence of the magnet. The first mistake occurred in the twenty-second trial (not on this record). Fish II had been trained in the opposite sense and was subjected to the same sequence of trials. The first mistake occurred in the sixteenth trial.

These results clearly indicate the suitability of *G. carapo* for training experiments; they further suggest (with the reservations made on p. 165) that the fish appreciates an electrical phenomenon and discriminates on this basis.

It seemed desirable to substantiate this view through experiments in which direct

contact of metals and the water could be avoided. Since these fish, like *Gymnarchus*, respond to the movement of a magnet outside their tank, an attempt was made to train two fish to a stationary magnet, placed behind a screen close to the food source. A strong horseshoe magnet was used for this purpose. The set-up and procedure was essentially as in the previous training experiments. One of the fish was trained to accept food in the presence of the magnet, and to refuse it for 30 sec. when the magnet had been removed. With the second fish the training procedure was reversed. Again, to avoid any possible subconscious optical signals by the experimenter, the final tests were carried out in complete darkness. The results of such a test are given in Text-fig. 10*b*. Although mistakes occurred, the fish were clearly aware of the presence or absence of the magnet behind the screen. Usually both fish developed a 'stereotyped' path of approach towards the food; mistakes appeared to occur more frequently when, for some reason, there was a deviation from this path.

In these experiments, it must be assumed, that the flow of water caused by the fish's movement, or the movement of the fish's body in the magnetic field, produces potential differences which are appreciated by *G. carapo* (see also Lissmann & Machin, 1958).

VI. DISCUSSION

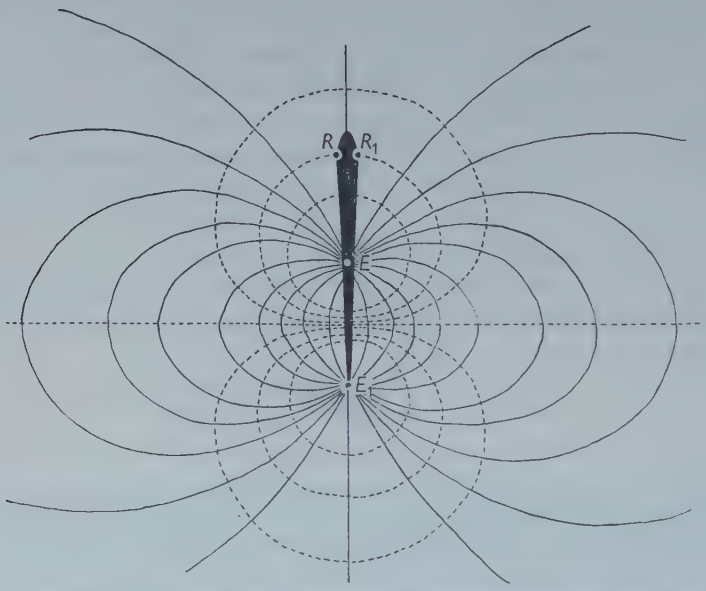
The theory of the function of electric organs suggested here implies that in a homogeneous aquatic environment the fish sets up an electric field which resembles that of an electrical dipole. Any object having a conductivity which differs from that of water may be expected to deflect the lines of force and will, if introduced into the water, disturb the original configuration of the field. It is assumed that the fish is able to sense this distortion of the field.

To test this theory a model was constructed consisting of two pairs of electrodes dipping into the water of a shallow tank. The first pair was connected to a beat-frequency oscillator and produced an electric dipole field comparable to that of a fish (Text-fig. 11). The second pair, corresponding to some hypothetical sense organs, was connected to an amplifier and an oscilloscope, and was placed at right angles to, and each member equidistant from, a line connecting the discharging electrodes. When both pairs of electrodes were carefully adjusted the recording electrodes were on a line of equipotential and no pulses were displayed on the oscilloscope. As soon as a piece of glass, metal or the human hand was now placed into the water in the vicinity of either electrode the pulses appeared on the oscilloscope. Also when a fish (non-electric) was introduced into the water its presence became visible on the oscilloscope whenever it approached the electrodes.

Although in a crude way this model may illustrate the mechanism used by electric fish, it also illustrates some of the difficulties: (i) both pairs of electrodes must be very carefully and rigidly adjusted to show the effect with any clarity; (ii) although the voltage of the discharges was higher than that recorded from weak electric fish, and although maximal gain was used on the recording side, the objects appeared detectable only at a relatively short range.

Nevertheless, it is useful to keep this model in mind when considering the possible anatomical and physiological counterparts in the fish body and the history of their origin.

While there may not be a single simple answer to the problem of the evolution of all electric organs, a number of unrelated forms have striking similarities in common. A comparative survey may, therefore, yield valuable information.



Text-fig. 11. Diagram of the electric field about equal charges of opposite sign. The lines of force (solid lines) and the lines of equipotential (dotted lines) are indicated. E and E_1 correspond to the discharging electrodes in the model or the electric organ of a fish; R and R_1 represent the recording electrodes or the hypothetical receptors.

(*a*) *The discharge mechanism*

All available evidence suggests that independent evolution of electric organs in fish has taken place at least seven times. In the majority of cases embryology and innervation indicate clearly that the origin is from muscular tissue. As far as is known there is nothing in fish muscles which may point to the reason why in this class of animals alone muscle should be particularly predisposed to such a specialization. Although the regularity of myotomic arrangement should make a reconstruction into serial electroplates perhaps more feasible, it must be remembered that not only tail and trunk muscles have been incorporated into electric organs—as in the case of Rajidae, Mormyridae, *Gymnarchus*, the Gymnotidae, and probably also in *Malapterurus* (Johnels, 1956)—but also hypobranchial muscles (Torpedinae) and even eye muscles (Astroscopus) (Dahlgren, 1927). The origin and significance of the electric organs in the submental filaments of *Staetogenes elegans* (Ellis, 1913; Lowrey, 1914) remains obscure. In an inquiry about the causes of the evolution of electric organs it appears more profitable to consider the whole functional complex

—as depicted in the model experiment—rather than attempt to find the reason in the fish muscle alone.

It has been mentioned that in the model experiment rigid fixation of the electrodes appears essential if the proposed mechanism is to work. In an active, living animal such degree of rigidity would seem to be difficult to achieve. Yet there are indications that in electric fish some provisions have been incorporated which achieve this to a certain extent. This seems obvious in forms like *Gymnarchus* and the Gymnotidae which during most phases of active movement can keep the electric organ in line with the long axis of the body. Forward and backward propulsion and turning movements can be accomplished in both types through the undulation of a very long unpaired fin. The fact that it is the dorsal fin in the case of *Gymnarchus*, and the anal fin in the case of all the Gymnotidae may be accepted as one of the many striking features of convergent evolution between these two groups (Text-fig. 5). The view, frequently expressed, that *Electrophorus* is reduced to swimming through undulations of the anal fin, because its 'trunk muscles' have been used up and transformed into electric organs, appears somewhat one sided since other gymnotids, despite their very small electric organs, have the same method of propulsion. At the same time this type of swimming mechanism does not *per se* imply an electrogenic fish; e.g. no discharges could be picked up from *Xenomystus nigri*.

In *Gymnarchus* and some Gymnotidae a complete loss of the tail fin has occurred. In other Gymnotidae a considerable reduction of the tail fin has taken place, and parallel instances can be found amongst the Mormyridae, such as *Isichthys henryi* and *Mormyrops attenuatus* (Text-fig. 5), which swim essentially through synchronous undulations of dorsal and anal fin, both much elongated. No counterparts are known amongst the Gymnotidae which would correspond in general body form and swimming movements to the large majority of the Mormyridae. These seem to propel themselves in an essentially piscine manner through lateral oscillations of the tail fin. The muscles, however, which move this tail fin lie further forward than in other fish and anterior to the electric organ; their tendons run over this organ. The region of the electric organ itself would appear to be stiffened to some extent against lateral bending through the four longitudinal 'Gemminger's bones' (sometimes fused). Two possible reasons for the function of these unique structures may be put forward: (i) since consecutive myotomes of the caudal peduncle have been weakened by incorporation of muscular tissue into the electric organ, this region may require the special skeletal support of these bones; (ii) if the symmetry of the electric field around the longitudinal axis of the fish is to be preserved during swimming movements, these bones may contribute towards the rigidity of this region of the body. It would not appear implausible to ascribe to 'Gemminger's bones' both these roles.

Apart from the fish examined here it may be significant that another family of electric fishes, the Rajidae, have an analogous propulsive mechanism. It will be recalled that in swimming these fish trail the rather rigid tail with its electric organ symmetrically throughout the locomotory cycles executed by the pectoral fins.

Torpedo, on the other hand, does swim by means of sculling motions of its tail

and makes no use of its pectoral fins for locomotion (Wilson, 1953). A similar mode of propulsion is used by *Astroscopus* (Dahlgren, 1927). Both these fish have cranial electric organs and the symmetry of the electric field will coincide with the symmetry of the fish's body except in the tail region. However, both are sluggish, bottom-living forms; if their—or their ancestor's—electric organ could be used for locating prey and other objects, the factor of maintaining a symmetrical field may not be so significant as in an actively hunting fish.

Whether similar speculations are applicable in the case of *Malapterurus* appears uncertain. The fish is not an active swimmer and when it swims it gives the appearance of a rather rigid sausage propelled by somewhat ostraciform tail movements. Three specimens of this species have been examined. None of them ever gave off the continuous electric pulses noted in the other species. When prodded they all produced a short train of strong, monophasic pulses (four to twenty). Otherwise they only discharged occasionally during feeding time, e.g. when a large worm was thrown into the water the fish would take it into its mouth, spit it out again, discharge, and then swallow the food. Nevertheless, it appears that a closer examination of the Siluridae would be profitable, because no intermediate forms between the strong electric *Malapterurus* and the other non-electric Siluridae have been found, a position which a few years ago had its close parallel in the Gymnotidae.

While one may thus assume that some mechanisms are incorporated in the general plan of these fish in order to maintain the symmetry of the electric field, these cannot be compared with the rigidity of the electrode system in the model. In fact, it can often be seen that the tip of the tail of a fish like *Gymnarchus*, when swimming backwards, may perform what looks like exploratory movements (see also Budgett (1901), p. 132, '*Gymnarchus* swims rapidly backwards...it may be seen to guide itself through the grasses by using this peculiar tail which it possesses as a feeler'). This type of movement is particularly noticeable in the early stages of training experiments when the fish often turns round and approaches the training site tail first, performing continuous 'scanning' motions with the tip of the tail. At this stage it is impossible to decide how far any proprioceptive information may be centrally integrated with electric information.

The intimate physiology and biochemistry of the discharges lies outside the scope of this paper. A considerable body of information has been accumulated in recent years (see Keynes & Martins-Ferreira, 1953; Grundfest, 1957), but insufficient comparative data are available on the mechanism of individual electroplates or their nervous control. The high frequency of discharges makes it appear unlikely that in all cases the discharge of an electroplate should be dependent on the arrival of a motor impulse.

In both the Gymnotidae and the Mormyriformes, two essentially different types of discharges have been found: (i) very regular, monophasic discharges of relatively long pulse duration and frequency independent of the state of excitation of the fish (*Adontosternarchus*, *Hypopomus*, *Eigenmannia*, *Gymnarchus*). The recorded frequencies vary between 60 and 940 impulses/sec. (ii) Complex di- or polyphasic pulses of more variable discharge rate and of very brief pulse duration (0·2 msec.

from the whole organ in *Petrocephalus*). Each pulse is separated from the next by a relatively long interval. The frequency of the discharges can increase considerably with excitation of the fish, and it can also be completely suppressed by suitable stimuli. The frequency range is between 0 and 200 impulses/sec.

At this stage no satisfactory explanation can be offered for the possible reasons behind these two discharge types, and no correlation with life habits has been established.

(*b*) *Receptor and integrating systems*

While the parallel between the discharges in the model and in the fish can be accepted, special difficulties are encountered in suggesting a suitable electrical receptor system which may serve as an analogue to the receiving electrodes in the model. On the other hand it must be pointed out that histologists for a long time have been at a loss in their efforts to find a suitable function for a number of sensory endings which are particularly common in electric fish or in closely related forms.

From a structural point of view the Mormyridae have been most carefully examined by a number of authors. Franz (1912, 1921), whose main conclusions have been rejected by later workers, has put forward an idea which is worth considering: 'A question for future research would be to consider whether perhaps the mormyrids themselves appreciate the electric shocks of members of their species, and thereby adjust their own behaviour; further whether the most peculiar structure of the epidermis affords protection for the internal organs against electric shocks' (Franz, 1921, pp. 140–1).

It is generally considered that in an electric field in fresh water the fish body acts like a relatively good conductor, enclosed in a less well-conducting membrane, surrounded by a bad conductor (Holzer, 1931, 1933; Spiecker, 1957). Although no experimental data appear to be available for Mormyridae, Franz's assumption that a thickening and layering of the epidermis would reduce the conductivity over the fish body does not seem unreasonable. As Marcusen (1864) has pointed out, this thick skin is rich in fat and contains many pores. Later examinations have shown (Stendell, 1914*a*, 1916; Franz, 1921; Berkelbach van der Sprenkel, 1915; Suzuki, 1932; Cordier, 1938; Gérard, 1940) that these pores lead through canals filled with a jelly-like substance to a variety of sense organs termed 'glandular sense organs' or 'mormyromasts'. These 'mormyromasts' are innervated by lateral line nerves and are particularly crowded at the snout and head region. They can be found over the whole body, except on the caudal peduncle, i.e. the region of the electric organ.* There appears little doubt that the enormous development of the 'mormyro-cerebellum' (enlarged valvulae cerebelli) can be correlated with the profusion of these sensory terminations. Conventional lateral line sense organs are also present in the Mormyridae but their numbers are unexceptional. Similar sensory endings have been described for *Gymnarchus* (Stendell, 1914*a*; Pehrson, 1945), and although the cerebellum does not reach the same dimensions as in the Mormyridae it is disproportionately large if compared with other fish.

* Wright (1958) claims to have located an electrical receptor at the base of the dorsal fin in a mormyrid. However, alternative interpretations of his experimental findings seem possible.

The Gymnotidae are less well documented in this respect. However, Coates, Cox & Smith (1938), while examining a papilloma of the skin in *Electrophorus*, state that the normal epidermis is rather thick, twenty to thirty cells in depth, resting upon a well-developed basement membrane. Extending outwards at right angles to the surface of the body are found at short, regular intervals numerous prolongations of fibrous tissue passing as supporting septa outwards into the epithelium. Coates (1950) mentions some enlarged pores of the lateral line around the head of this fish. Although he states that he has been 'unable to find anything of particular interest beneath these pores' he suggests a connexion of some cranial receptors and the locating mechanism, since insulation of the head upset the orientation of a fish. His view that electro-magnetic waves, analogous to radar, are at work and that the fish is able to appreciate the time delay of outgoing and reflected waves (Coates, 1947) over a distance of a few inches appears unacceptable on physical and physiological grounds. Again, the cerebellum in gymnotids is a massive organ, but the neuro-histology is not sufficiently known in detail. The picture as a whole presented by the Gymnotidae, despite the scanty information of finer detail, does not appear unlike that of the Mormyridae. A comparative study of gymnotid brains and receptors would be clearly of great interest. The parallel evolution of electric organs and long snouts in both families (Text-fig. 5) tempts one to predict the occurrence of 'Schnauzenorgane' (Stendell, 1916) in both.

The first discussion of the possibility of an 'electric sense' appears to be that which arose from a misunderstanding of Wagner's (1847) paper by Boll (1873, 1875) and concerns Savi's vesicles in *Torpedo*. These completely encapsulated, round sense organs, 2 to 3 mm. in diameter, are found in great numbers, regularly spaced, beginning at the outer perimeter of the electric organ and extending to the snout and pectoral fins. They form part of the lateralis system of these fish. Boll rejects them as possible 'electric sense organs' because (i) electrical stimulation of the proximal nerve supplying these organs 'excites the activity of the electric organ neither more nor less than stimulation of any other sensory nerve'; (ii) analogous organs in the two other species of electric fish are not known.

Neither of these two arguments appears acceptable to-day. It is interesting to note that not only are the eyes poorly developed in most species of *Torpedo*, as they are in *Electrophorus* and *Gymnarchus*, but that a number of blind species are known with relatively smaller electric organs (e.g. *Typhlonarke*, Garrick, 1951).

The small, essentially serial caudal electric organ of the Rajidae has hardly been investigated from a biological point of view, although it can be made to discharge in a physiological experiment. The idea that the fish's prey may be stunned by such electric shocks is hardly tenable since many species are mollusc feeders. On the other hand the differentiation of the lateralis system, notably the ampullae of Lorenzini (also found in *Torpedo*) have attracted much attention. These grape-like vesicles are located some distance beneath the surface of the body and are in communication with the outside through long tubes, filled with a jelly-like substance. The 'mormyromasts' have often been likened to the ampullae of Lorenzini. After much inconclusive speculation and experimentation the balance of opinion

to-day has shifted from the 'pressure receptor theory' towards the 'temperature receptor theory' (Sand, 1938; Hensel, 1955). Observations of the impulses along the afferent nerves of these organs have shown that they behave similarly to mammalian cold-receptors, and they have been shown to respond to temperature changes of 0·05° C. Before, however, accepting this interpretation as satisfactory three points should be cleared up: (i) why should a sensitive temperature receptor be located deep in the body, when it takes several seconds to transmit the temperature to that depth? (ii) The cranial innervation and the divergence of the canals from these organs suggest a receptor designed for a rapid point-to-point correlation of outside stimuli. It is difficult to see why temperature receptors should occupy such an exceptional position in the skate. (iii) What is the biological stimulus to which these organs are adapted to respond?

It is interesting that Franz (1912, p. 487), in discussing the cerebellum of fishes, notes that the mormyrid cerebellum—and also for unknown reasons the cerebellum of the sluggish skates—is disproportionately large. Even if the mode of subdivision of the cerebellum is different in teleosts and elasmobranchs, and the valvula cerebelli not typically represented in the latter, the pars auricularis ('restiform bodies') forms a corresponding centre (Kappers, 1906; Herrick, 1924); this part is said to be particularly well developed in the skates.

The main difficulty in accepting the ampullae of Lorenzini as electric sense organs derives from the fact that some fish are endowed with these sense organs without, apparently, possessing specific electric organs. This applies not only to elasmobranchs but equally to the Siluridae. Only one member of this family, *Malapterurus*, is known to possess an electric organ. Yet Siluridae are the only bony fishes known to possess typical ampullae of Lorenzini (Friedrich-Freska, 1930). The species *Plotosus anguillaris*, in which these organs have been discovered, is, perhaps significantly, one of the few marine Siluridae. In freshwater catfishes Herrick (1901, 1903) has discovered the 'small pit organs'. He states that 'the aperture by which the pit communicates with the surface is a very minute pore'. . . and 'the pore may run downward as a straight tube of considerable length before dilating into the sac-like cavity of the pit'. Again, these organs are innervated by lateralis nerves and glandular or mucous cells are found in close proximity to the sense organs. The resemblance to 'mormyromasts' is unmistakable.

Moreover, although these catfishes do not possess an electric organ their great sensitivity towards electrical stimulation has been noted by several observers (Parker & van Heusen, 1917; Hatai, Kokubo & Abe, 1932; Abe, 1935; Kokubo, 1934; Uzuka, 1934); Parker & van Heusen (1917) also note that these fish respond to an electro-magnet.

Another significant feature seems to be the fact that next to the Mormyridae, the Siluridae show the most specialized development of the valvula cerebelli (Kappers, 1906; Berkelbach van der Sprenkel, 1915).

No comparable data seem to be available for *Astroscopus*; it is interesting, however, that larval pelagic specimens do not possess electric organs. The transformation of the eye muscles into electric organs can be related to the change to a benthonic

life habit in the adult fish which has a number of striking adaptations suggesting an environment of poor visibility (Dahlgren, 1927).

(*c*) *General considerations of the lateralis system*

Although the general picture of the evolution of electric organs is by no means clear to date, the crowding of coincident features does not appear without significance. It may seem that the possession of suitable receptors, rather than the ability to transform muscles into electric organs, is the major prerequisite for this step in evolution.

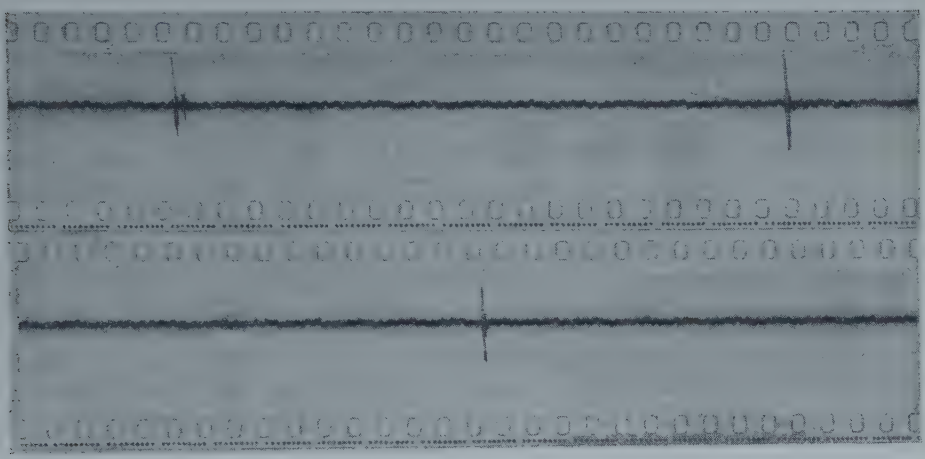
It is therefore of interest to consider the properties and mode of action of the lateral line sense organs in their more generalized form. The fact that they serve as mechano-receptors seems to be universally accepted (Dijkgraaf, 1933, 1952; Sand, 1937; Katsuki, Yoshino & Chen, 1950, 1951 *a*, *b*). The transformation of such a receptor, which in its most primitive form projects over the surface of the body and is covered by a jelly-like cupola, into an electro receptor which is sunk below the surface, may appear a formidable difficulty. Even if we accept the perception of small water currents, vibrations and pressures as the primary function of the superficial lateralis organs, and also the reasons advanced by Dijkgraaf (1952) for their subsequent inclusion into lateral line canals, it must be remembered that Regnart (1931) has shown that the lower limits of perception of electric currents by fish (codling) are dependent on the presence of such lateral line organs.*

Given the ability to respond to electric stimuli the next question which arises would be as to the nature and origin of electrical stimuli any pre-electric fish may encounter. In this respect our knowledge is very incomplete, but one source which could be suggested is the muscular action potential of prey, predator, members of the same species and of the fish itself†. In an aquatic environment the recording of such action potentials some distance away from the animal presents no special difficulties. Text-fig. 12 shows such electrical disturbances recorded from an eel (*Anguilla vulgaris*, 38 cm. long) in a shallow tank. In the middle of the tank were placed two bricks between which the fish was sheltering. Electrodes, 12 cm. apart and 1 cm. long, were fixed more or less parallel to the posterior end of the animal and 10 cm. from it. Whenever a hand was passed over the tank, casting a shadow, the eel performed a jerk, and what appeared to be a muscular action potential was displayed on an oscilloscope. This procedure could be repeated up to ten or twelve times in rapid succession, after which the animal ceased to respond. After a short while the same phenomenon could be evoked again. It can be imagined, particularly in a gregarious species, that the perception of such electrical disturbances could be of survival value. Recordings of spike potentials, coincident with the breathing movements of *Petromyzon marinus*, have also been picked up from the surrounding

* I am aware that these findings have not been universally accepted, e.g. Spiecker (1957). Regnart used nerve section, Spiecker superficial application of anaesthetics to eliminate this sense; moreover, Regnart is concerned with the 'primary reaction' (lower threshold) and not with 'galvanotaxis'.

† Hoagland (1935) considers the possible role of the lateral line as proprioceptor.

water by Kleerekoper & Sibakin (1956). We have no reason for believing that our electronic recording devices may be superior to a fishes receptor. Whether fish, having evolved great sensitivity towards electrical stimuli, may respond to other types of electrical phenomena of a magnitude likely to arise in nature, will be considered in another paper (Lissmann & Machin, 1958).



Text-fig. 12. Muscular action potentials recorded from an eel (*Anguilla vulgaris*) at a range of about 10 cm.; these could be observed when the resting fish, in response to visual stimulation, performed a sudden movement. Time marker 0·1 and 0·01 sec.

In the absence of any precise experimental evidence one can only surmise that, like other acoustico-lateralis sense organs, such electro-receptors will show a resting discharge, and that the frequency of the impulses may be varied by ascending and descending electric currents (Löwenstein, 1953). In the course of evolutionary transformation a stage of double specificity—to mechanical and electrical stimuli—must be postulated. The details of the mechanism by which the sensory cells of the labyrinth and the lateral line are excited are still not perfectly understood, but the microphonic potentials show a close association of mechanical and electrical events. The view that such voltages are generated by piezo-electric properties of the cupola seem to have been abandoned (Jielof, Spoor & de Vries, 1952). On the other hand, it has been suggested that the endolymph may play a significant role (Jensen, Koefoed & Vilstrup, 1954), since it contains a fair amount of hyaluronic acid. This solution, when mechanically displaced in a tube, sets up electrical flow potentials. Hyaluronic acid is also present in the jelly which fills the lateral line canals of fish (Katsuki, Mizuhira & Yoshino, 1952), though apparently not in the cupolae of lateral line receptors. Whether the jelly-like substance which fills the tubes leading to 'mormyromasts' and similar sense organs contains solutions exhibiting the phenomenon of flow potentials is not known. In view of the electrical nature of the stimulus there appears no need to postulate any special properties of this substance except electrical conductivity.

(*d*) *Conclusion*

In the absence of any existing, coherent theories about the evolution of electric organs, and about the function of weak electric organs, the speculative picture presented here may fill a gap.

It is imagined that the first essential feature in the process of evolution of electric organs was the possession of receptors sensitive to electric stimuli. At an early stage this sensitivity may be regarded as an incidental, later a subsidiary and finally the specific function of such sense organs. On structural and functional grounds the lateral line receptors seem to fit the requirements best. Parallel with their differentiation the evolution of electric organs from muscular tissue may be postulated. The muscular action potential can be assumed to have been an initial stimulus source. Environmental conditions which can be expected to favour this trend in evolution would be those that preclude the normal functioning of other receptors (e.g. turbidity of water).

The weak electric fish discussed here represent already very advanced, highly specialized forms. The theory of the use of their electric organ offered in this paper suggests that these fish are able to use the information arising from distortions—caused by various outside sources—of the electric field which they themselves so regularly produce. The fact that electric fish have shown themselves extremely sensitive to minute electric influences produced experimentally, and that such experiments could not be repeated with non-electric fish, indicates that the great sensitivity of electric fish is related to the emission of electric discharges. Both on the effector and receptor side the central nervous system shows great specializations. The nervous mechanisms of the effector component have been recently explored in various species (Fessard & Szabo, 1953; Szabo, 1954, 1955). The 'mormyrocerebellum' and similar structures can be considered essentially as the relevant sensory integrating systems.

However, this specialization, once it has begun, does not only involve the organs of this one reflex arc. New locomotory mechanisms, new epidermal adaptations to electrical conductivity, etc., may follow, so that finally it can be imagined that such a fish, living in a private, electric world of its own, receives a variety of information through sense organs distributed over the surface of its body which may be likened to an 'electro-receptive retina'. The effectiveness of stimuli impinging on this retina can be expected to be enhanced if the epidermis in which they are embedded has improved insulating properties.

The striking elongation of the body in some freshwater forms can, in part, be regarded as an expression of the tendency to extend the effective range of this mechanism, e.g. the viscera proper occupy a space a little longer than the length of the head in many Gymnotidae (Text-fig. 5); they can be as short as one-eighth of the total length with the position of the anus anterior to the level of the eyes (Schlesinger, 1910). As the number of serial electric elements is increased, and thereby the voltage of the discharge, so a stage will be reached when 'objects', which previously could only be located, can now be stunned and swallowed.

In many details the views expressed in this paper can be subjected to a critical test by anatomical, histological and experimental investigation. On the outcome must depend the acceptance or rejection, in whole or in part, of the theories which are here tentatively suggested.

In the course of this work I have received much help and advice from friends and colleagues too numerous to mention. I am particularly grateful to Prof. Sir James Gray for his interest, support and patience throughout this period. In Africa I have enjoyed the hospitality of Dr and Mrs K. R. S. Morris, without whose expert knowledge much of this work could not have been done. I also have to thank Prof. and Mrs E. E. Edwards and their staff at the Department of Zoology, University College, Achimota, for hospitality and assistance. Financial aid from the Balfour Fund made the expedition to Ghana possible. Mr J. A. Popple has given me much technical assistance in the early stages of this work, and Mr H. R. Klose has, throughout, devoted much care and attention to the fish. My thanks are also due to Dr Sabet Girgis, who has supplied me with specimens from the Sudan, and to Mrs R. H. McConnell for efficiently organizing the supply of material from British Guiana.

SUMMARY

1. The electric discharges of *Gymnarchus niloticus* and of representative species of seven genera of the Mormyridae have been examined in their natural habitat in Africa and in the laboratory.

2. Comparable investigations of the South American Gymnotidae have shown the existence of two discharge types in both these unrelated fish families.

3. The first type of electric discharge consists of very regular sequences of continuously emitted, monophasic pulses, varying from species to species in frequency, and within narrower limits from individual to individual.

4. Fish emitting this first type of pulses include *Gymnarchus*, *Hypopomus* and *Eigenmannia*. The frequency range for these fish lies between 60 and 400 discharges/sec.

5. The frequency does not alter with the state of excitation of the fish. The duration of individual pulses is relatively long, i.e. 2–10 msec.

6. The second type of discharge is less regular in frequency, the pulse duration much shorter and the pulse shape more complex. The individual discharge from the whole electric organ lasts about 0·2 msec. in *Petrocephalus*.

7. This type of discharge is found in all the examined species of the Mormyridae and in such forms as *Gymnotus carapo* and *Staetogenes elegans*.

8. The basic discharge rate of a resting mormyrid is somewhat variable and not strictly rhythmical. It usually lies between 1 and 6 pulses/sec.

9. Stimuli which excite the mormyrids cause an increase in the discharge frequency. The recorded maximum is about 130 pulses/sec.

10. Suitable stimuli can inhibit the discharges of the Mormyridae for prolonged periods.

11. In *Gymnotus carapo* and *Staetogenes elegans* the basic discharge rate is higher and of regular rhythmicity. Depending on temperature the frequencies lie between 30 and 87 pulses/sec. When these fish are excited the frequencies are increased up to 200 pulses/sec. for a short time.

12. The shape of the electric field, which is set up with each pulse around the fish, has been examined.

13. A theory has been proposed which suggests that these fish, by means of their electric pulses, can locate objects if their electrical conductivity differs from that of water.

14. These fish have shown themselves extremely sensitive to influences affecting the electric field. This has been studied by applying artificial electric stimuli, by studying the effects of conductors and non-conductors introduced into the field, and the reactions towards magnetic fields and electrostatic charges.

15. Conditioned reflex experiments with *Gymnarchus niloticus* and *Gymnotus carapo* have shown that these fish can detect the presence of a stationary magnet, and that they can discriminate between conductors and non-conductors.

16. The prey of these fish does not appear to be affected by the discharges. *Inter alia*, the electric pulses have a social significance.

17. This locating mechanism may be considered as an adaptation to life in turbid water.

18. Gymnotidae and Mormyridae (taken to include *Gymnarchus*) show striking features of convergent evolution.

19. Unusual locomotory adaptations such as swimming by means of the dorsal fin (*Gymnarchus*), the anal fin (Gymnotidae) and 'Gemminger's bones' (Mormyridae) may be considered as a means which tends to make the axis of symmetry of the fish and of its electric field coincide during active movements.

20. A new theory for the evolution of electric organs has been suggested. A major prerequisite appears to be a receptor sensitive to electrical stimulation.

21. It is suggested that special sensory and nervous differentiations of the lateralis system ('mormyromasts', valvulae cerebelli) are concerned with the perception and integration of electric stimuli.

22. Muscular action potentials have been recorded in the water at some distance from non-electric fish.

23. The easiest explanation for the evolution of strong electric organs would appear to start from such muscular action potentials, and proceed via weak electric organs used for orientation, to the powerful offensive and defensive electric organs.

REFERENCES

ABE, N. (1935). Galvanotropism of the catfish *Parasilurus asotus* (Linné). *Sci. Rep. Tôhoku Univ* (*d*) **9**, 393–406.

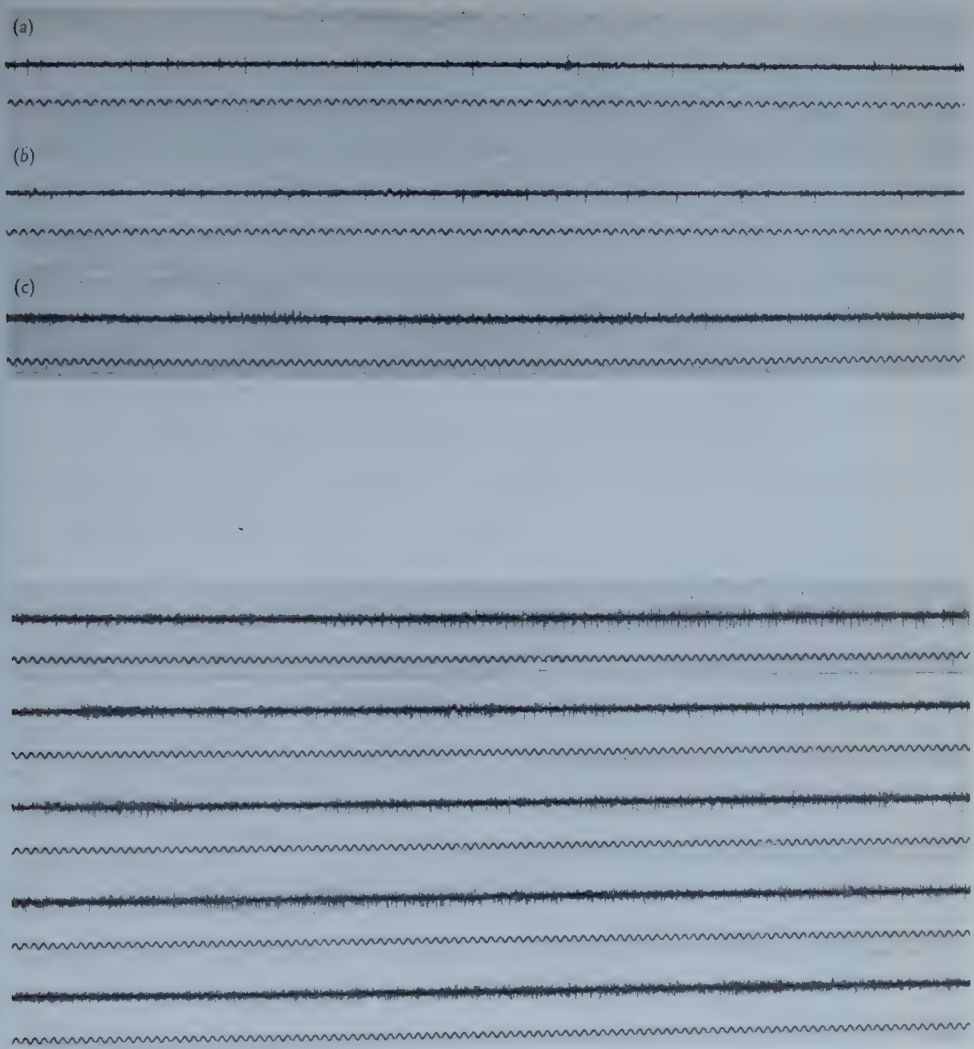
ASHETON, R. (1907). The development of *Gymnarchus niloticus*. The Work of John Samuel Budgett, pp. 293–422, ed. J. G. Kerr. Cambridge.

BABUCHIN, A. I. (1872). Über die Bedeutung und Entwicklung der pseudo-elektrischen Organe. *Zbl. med. Wiss.* **10**, 545–8.

BABUCHIN, A. I. (1877). Beobachtungen und Versuche am Zitterwels und *Mormyrus* des Nils. *Arch. Anat. Physiol., Lpz.*, 250–74.

Berg, L. S. (1947). *Classification of Fishes both Recent and Fossil.* Michigan.
Berkelbach van der Sprenkel, H. (1915). The central relations of the cranial nerves in *Silurus glanis* and *Mormyrus caschive*. *J. Comp. Neurol.* **25**, 1–63.
Boll, F. (1873). Beiträge zur Physiologie von *Torpedo*. *Arch. Anat. Physiol., Lpz.*, 76–102.
Boll, F. (1875). Die Savischen Bläschen von *Torpedo*. *Arch Anat. Physiol. Lpz.*, 456–468.
Boulenger, G. A. (1904). Übersicht der Unterordnungen und Familien der Teleostier. *Arch. Naturgesch.* **1**, 197–228.
Boulenger, G. A. (1909). *Catalogue of the Fresh-water Fishes of Africa in the British Museum*, vol. 1. London.
Budgett, J. S. (1901). On the breeding habits of some West African fishes, with an account of the external features in development of *Protopterus annectens*, and a description of the larva of *Polypterus lapardi*. *Trans. Zool. Soc. Lond.* **16**, 115–36.
Coates, C. W. (1947). The kick of an electric eel. *Altant. Mon.* **180**, 75–79.
Coates, C. W. (1950). Electric fishes. *Electr. Engng*, 1–4.
Coates, C. W., Altamarino, M. & Grundfest, H. (1954). Activity in electrogenic organs of knifefishes. *Science*, **120**, 845–6.
Coates, C. W. (1955). Three more gymnotid eels found to be electrogenic. *Zoologica, N.Y.*, **40**, 197–8.
Coates, C. W., Cox, R. T. & Smith, G. M. (1938). Papilloma of the skin in the electric eel, *Electrophorus electricus*. *Zoologica, N.Y.*, **23**, 247–51.
Cordier, R. (1938). Sur les organes sensoriel cutanés du Mormyridé *Gnathonemus monteiri*. *Ann. Soc. zool. Belg.* **68**, 77–90.
Couceiro, A., Leao, A. A. P. & Castro, G. O. (1955). Some data on the structure of the electric organ of the Itui, *Sternarchus albifrons* (L). *Ann. Acad. bras. Cien.* **27**, 323–8.
Dahlgren, U. (1910). Origin of the electricity tissues in fishes. *Amer. Nat.* **44**, 193–202.
Dahlgren, U. (1914). Origin of electric tissues of *Gymnarchus niloticus*. *Publ. Carneg. Instn*, **183**, 159–94.
Dahlgren, U. (1927). The life history of the fish *Astroscopus* (the 'Stargazer'). *Sci. Mon., N.Y.*, **24**, 348–65.
Darwin, Ch. (1872). *The Origin of Species by Means of Natural Selection*, 6th ed. London.
Dijkgraaf, S. (1934). Untersuchungen über die Funktion der Seitenorgane bei Fischen. *Z. vergl. Physiol.* **20**, 162–214.
Dijkgraaf, S. (1952). Bau und Funktionen der Seitenorgane und des Ohrlabyrinths bei Fischen. *Experientia*, **8**, 205–16.
Ellis, M. M. (1913). The Gymnotid Eels. *Mem. Carneg. Mus.* **6**, 109–95.
Erdl, M. P. (1846). Über eine neue Form elektrischen Apparates bei *Gymnarchus niloticus*. *Bull. bayer. Akad. Wiss.* (*Gelehrte Anzeigen*), **24**, 585–9.
Erdl, M. P. (1847). Beschreibung des Skelets von *Gymnarchus niloticus*, nebst Vergleichung mit Skeletten formverwandter Fische. *Abh. bayer. Akad. Wiss.* **5**, 209–52.
Fessard, A. & Szabo, Th. (1953). Sur l'organisation anatomofonctionelle des lobes électriques de la Torpille. *J. Physiol. Path. gén.* **45**, 114–17.
Franz, V. (1912). Das Mormyridenhirn. *Zool. Jb.* (Abt. 2), **32**, 465–92.
Franz, V. (1921). Zur mikroskopischen Anatomie der Mormyriden. *Zool. Jb.* (Abt. 2), **42**, 91–148.
Friedrich-Freska, H. (1930). Lorenzinische Ampullen bei dem Siluriden *Plotosus anguillaris* Bloch. *Zool. Anz.* **87**, 49–66.
Fritsch, G. (1885). Zur Organisation des *Gymnarchus niloticus*. *S.B. preuss. Akad. Wiss.* **42**, 119–29.
Fritsch, G. (1891). Weitere Beiträge zur Kenntnis der schwach elektrischen Fische. *S.B. preuss. Akad. Wiss.* **42**, 941–62.
Garrick, J. A. F. (1951). The blind electric rays of the genus *Typhlonarke* (Torpedinidae). *Zool. Publ. Vict. Univ. N.Z.* No. 15, 1–6.
Gérard, P. (1940). Sur les appareils sensoriel de la peau de *Mormyrus caballus*. *Bull. Inst. Roy. Colon. Belge*, **11**, 212–26.
Grundfest, H. (1957). The mechanism of discharge of the electric organs in relation to general and comparative electro physiology. *Progr. Biophys.*, **7**, 1–86.
Hatai, S., Kokubo, S. & Abe, N. (1932). The earth currents in relation to the response of catfish. *Proc. Imp. Acad. Japan*, **8**, 478–81.
Hensel, H. (1955). Über die Funktion der Lorenzinischen Ampullen der Selachier. *Experientia*, **11**, 325.
Herrick, C. J. (1901). The cranial nerves and cutaneous sense organs of the North American siluroid fishes. *J. Comp. Neurol.* **11**, 172–249.
Herrick, C. J. (1903). On the morphological and physiological classification of the cutaneous sense organs of fishes. *Amer. Nat.* **37**, 313–18.

HERRICK, C. J. (1924). Origin and evolution of the cerebellum. *Arch. Neurol. Psychiatr.* **11**, 621–52.
HOAGLAND, H. (1935). *Pacemakers in Relation to Aspects of Behaviour.* New York.
HOLZER, W. (1931). Über die absolute Reizspanne bei Fischen. *Pflüg. Arch. ges. Physiol.* **229**, 153–72.
HOLZER, W. (1933). Modelltheorie über die Stromdichte im Körper von Lebewesen bei galvanischer Durchströmung in Flüssigkeit. *Pflüg. Arch. ges. Physiol.* **232**, 835–41.
HYRTL, J. (1856). Anatomische Mitteilungen über *Mormyrus* und *Gymnarchus*. *Denkschr. Akad. Wiss. Wien*, **12**, 1–22.
JENSEN, C. E., KOEFOED, J. & VILSTRUP, T. (1954). Flow potentials in hyalurate solutions. *Nature, Lond.*, **174**, 1101.
JIELOF, R., SPOOR, A. & DE VRIES, H. (1952). The microphonic activity of the lateral line. *J. Physiol.* **116**, 137–57.
JOHNELS, A. F. (1956). On the origin of the electric organ in *Malapterurus electricus*. *Quart. J. micr. Sci.* **97**, 455–64.
KAPPERS, C. U. A. (1906). The structure of the teleostean and selachian brain. *J. Neurol. Comp.* **16**, 1–109.
KATSUKI, Y., MIZUHIRA, V. & YOSHINO, S. (1952). On the endorgan of the acoustico-lateralis system in fish. *Jap. J. Physiol.* **2**, 93–102.
KATSUKI, Y., YOSHINO, S. & CHEN, J. (1950). Action currents of the single lateral-line nerve fibre of fish. I. On the spontaneous discharge. *Jap. J. Physiol.* **1**, 87–99.
KATSUKI, Y., YOSHINO, S. & CHEN, J. (1951*a*). Action current of the single lateral-line nerve fibre in fish. *Jap. J. Physiol.* **1**, 179–94.
KATSUKI, Y., YOSHINO, S. & CHEN, J. (1951*b*). Neural mechanism of the lateral line organ of fish. *Jap. J. Physiol.* **1**, 264–8.
KEYNES, R. D. & MARTINS-FERREIRA, H. (1953). Membrane potentials in the electroplate of the electric eel. *J. Physiol.* **119**, 315–50.
KLEEREKOPER, H. & SIBAKIN, K. (1956). An investigation of the electrical spike potentials produced by the sea lamprey (*Petromyzon marinus*) in the water surrounding the head region. *J. Fish. Res. Bd, Can.* **13**, 375–83.
KOKUBO, S. (1934). On the behaviour of catfish in response to galvanic stimuli. *Sci. Rep. Tôhoku Univ.* (*d*), **9**, 87–96.
LISSMANN, H. W. (1951). Continuous electrical signals from the tail of a fish, *Gymnarchus niloticus* Cuv. *Nature, Lond.*, **167**, 201.
LISSMANN, H. W. & MACHIN, K. E. (1958). The mechanism of object location in *Gymnarchus niloticus* and similar fish. *J. Exp. biol.* (in the Press).
LÖWENSTEIN, O. (1953). The effect of galvanic polarization on the impulse discharge from the horizontal ampulla of the isolated elasmobranch labyrinth. *Nature, Lond.*, **172**, 549.
LOWREY, A. (1914). A study of the submental filaments considered as probable electric organ in the gymnotid eel *Staetogenes elegans* (Steindachner). *J. Morph.* **24**, 685–94.
MARCUSEN, J. (1864). Die Familie der Mormyren. Eine anatomisch-zoologische Abhandlung. *Mém. Acad. Sci. St Pétersb.* **7**, 1–162.
MOEHRES, F. P. (1957). Elektrische Entladungen im Dienste der Revierabgrenzung. *Naturwissenschaften*, **44**, 431–2.
OGNEFF, J. (1898). Einige Bemerkungen über den Bau des schwachen elektrischen Organs bei den Mormyriden. *Z. wiss. Zool.* **64**, 565–95.
OMARKHAN, M. (1949). The morphology of the chondrocranium of *Gymnarchus niloticus*. *J. Linn. Soc.* **41**, 452–81.
PARKER, G. H. & VAN HEUSEN, A. P. (1917). The responses of the catfish, *Amiurus nebulosus*, to metallic and non-metallic rods. *Amer. J. Physiol.* **44**, 405–20.
PEHRSON, T. (1945). The system of pit organ lines in *Gymnarchus niloticus*. *Acta zool, Stockh*, **26**, 1–8.
REGAN, C. T. (1911). The classification of teleostean fishes of the order Ostariophysi. *Ann. Mag. Nat. Hist.* **8**, 13–32.
REGNART, H. C. (1931). On the lower limits of perception of electrical currents by fish. *J. Mar. Biol. Ass. U.K.* **17**, 415–20.
RIDEWOOD, W. (1904). The cranial osteology of the fishes of the families Mormyridae, Notopteridae and Hydontidae. *J. Linn. Soc.* **29**, 188–201.
RÜPPEL, E. (1832). *Fortsetzung der Beschreibung und Abbildung mehrerer neuen Fische im Nil entdeckt.* Frankfurt a.M.
SAND, A. (1937). Mechanism of the lateral sense organs of fishes. *Proc. Roy. Soc.* B, **123**, 472–95.
SAND, A. (1938). The function of the ampullae of Lorenzini with some observations on the effect of temperature on sensory rhythms. *Proc. Roy. Soc.* B, **125**, 524–53.



LISSMANN—FUNCTION AND EVOLUTION OF ELECTRIC ORGANS IN FISH

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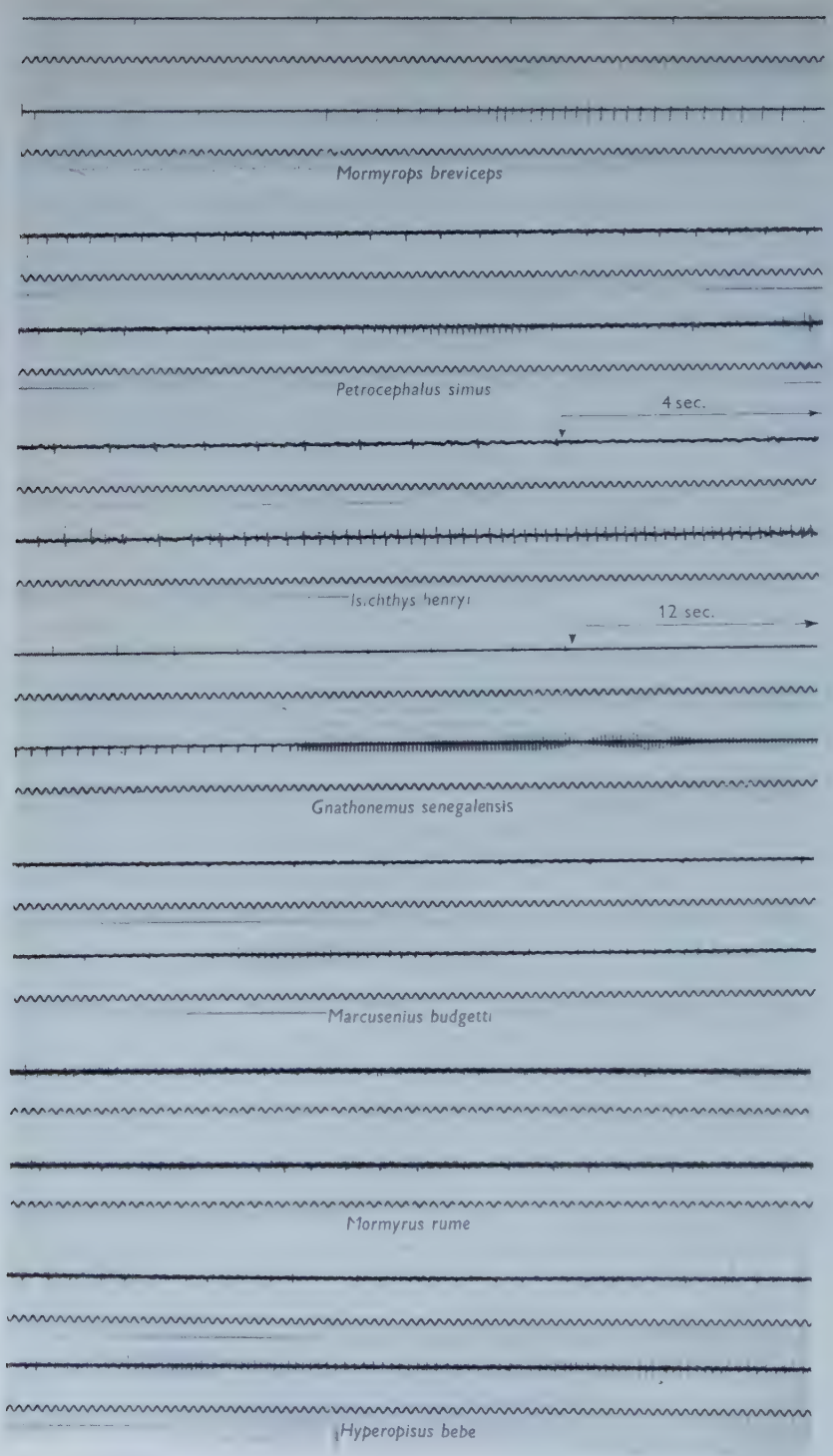

LISSMANN—FUNCTION AND EVOLUTION OF ELECTRIC ORGANS IN FISH

Sanders, A. (1882). Contributions to the anatomy of the central nervous system in vertebrate animals. *Phil. Trans.* **173**, 927–59.
Sanderson, J. B. & Gotch, F. (1888). On the electrical organ of the skate. *J. Physiol.* **9**, 137–66.
Schaffer, E. L. (1917). On the electric organ of *Gymnotus carapus*. *Science*, **45**, 67–9.
Schlesinger, G. (1909). Zur Ehtologie der Mormyriden. *Ann. naturh.* (*Mus.*) *Hofmus., Wien*, **23**, 282–311.
Schlesinger, G. (1910). Die Gymnotiden. Eine phylogenetisch-ethologische Studie. *Zool. Jb.* (Abt. 1), **29**, 613–40.
Schlichter, H. (1906). Über den feineren Bau des schwach-elektrischen Organs von *Mormyrus oxyrhynchus*, Geoffr. *Z. wiss. Zool.* **84**, 479–525.
Spiecker, D. (1957). Die Galvanotaxis der Fische und ihre Auslösung auf Grund unmittelbarer Irritabilität der Rumpfmuskulatur. *Zool. Jb.* (Abt. 3), **67**, 229–60.
Stendell, W. (1914*a*). Morphologische Studien an Mormyriden. *Verh. dtsch. zool. Ges.* **24**, 254–61.
Stendell, W. (1914*b*). Die Faseranatomie des Mormyridenhirns. *Abh. senckenb. naturf. Ges.* **36**, 1–39.
Stendell, W. (1916). Die Schnauzenorgane der Mormyriden. *Z. wiss. Zool.* **115**, 650–69.
Suzuki, N. A. (1932). A contribution to the study of the mormyrid cerebellum. *Annot. zool. Jap.* **13**, 503–24.
Svensson, G. S. O. (1933). Freshwater fishes from the Gambia River (British West Africa). Results of the Swedish Expedition 1931. *K. svenska VetenskAkad. Handl.* **12**, 1–102.
Szabo, Th. (1954). Un relais dans le système des connexions du lobe électrique de la Torpille. *Arch. Anat. micr. Morph. exp.* **43**, 187–201.
Szabo, Th. (1955). Quelques précisions sur le noyau de commande centrale de la décharge électrique chez la Raie (*Raja clavata*). *J. Physiol. Path. gén.* **47**, 283–5.
Szabo, Th. (1957). Le muscle d'origine de l'organe electrique d'un Mormyre (*Gnathonemus senegalensis elongatus*). *Z. Zellforsch.* **47**, 77–79.
Uzuka, K. (1934). Some notes on the behaviour of the catfish, *Parasilurus asotus*, as seen through the responses to weak electric current. *Sci. Rep. Tôhoku Univ.* (*d*) **8**, 369–81.
Wagner, R. (1847). Über den feineren Bau des elektrischen Organs im Zitterrochen. *Abh. Ges. Wiss. Göttingen.* **3**, 141–66.
Wilson, D. P. (1953). Notes from the Plymouth Aquarium. *J. Mar. Biol. Ass. U.K.* **32**, 199–208.
Wright, P. G. (1958). An electrical receptor in fishes. *Nature, Lond.*, **181**, 64–5.

EXPLANATION OF PLATES 5 AND 6

Plate 5

1. Electric discharges recorded in African rivers (Black Volta (*a*, *b*), Kamba (*c*)). (*a*) The record indicates apparently three resting specimens discharging at a slow rhythm, of about 3 to 4 pulses/sec. (*b*) The impulses of a single, resting specimen which showed periodic acceleration of the discharges. (*c*) Pulses of frequencies of 20–50/sec. usually remain in evidence only for a short time and suggest an actively swimming specimen. Time marker 50 cyc./sec.

2. Recording of electric discharges from a small pool in the dry season. It contained species of *Petrocephalus*, *Marcusenius*, *Gnathonemus* and *Hyperopisus*. The discharges remained in evidence throughout the period of observation. Time marker 50 cyc./sec.

Plate 6

Discharges of representatives of seven genera of the Mormyridae, first at rest (upper record) then excited (lower record). Note the great increase in the frequency of the discharges with excitation. The upper and lower records are continuous in each case, except for *Gnathonemus senegalensis* and *Isichthys henryi*. In the former there was a period of 12 sec. during which the fish did not discharge while the experimenter approached the aquarium and was leaning over it before touching the fish with a glass rod. Similarly, *I. henryi* did not discharge for 4 sec. Note also the grouping of discharges into pairs prior to stimulation of *Hyperopisus bebe*; this has also been recorded in the rivers both during slow and fast discharge frequencies. Time marker 50 cyc./sec.

CHEMOTAXIS OF BRACKEN SPERMATOZOIDS

THE ROLE OF BIMALATE IONS

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Fern spermatozoids respond chemotactically to concentration gradients of malic or maleic acid salts (Pfeffer, 1884). They respond weakly to salts of a few other dicarboxylic acids, but do not respond to succinate or fumarate (Shibata, 1911; Rothschild, 1956). Previous workers concluded, without adequate justification, that the malate ion, and not the undissociated malic acid molecule, is chemotactically active; but no consideration has been given to the possibility that the bimalate ion may be the active species.

A malate-dependent aggregation of bracken spermatozoids in a pH gradient has been briefly reported (Brokaw, 1957). In this paper, this phenomenon and several related observations are examined in greater detail.

MATERIAL AND METHODS

Spores of bracken, *Pteridium aquilinum* (L.) Kuhn, were sown thickly on agar plates made up with approximately 1% agar in a Knop's solution (0·1% KNO_3, 0·05% NaCl, 0·05% $CaSO_4$, 0·05% $MgSO_4$, 0·05% $Ca_3(PO_4)_2$). A dense mat of small prothalli was obtained after about 3 months' growth. When a piece of this mat is removed, placed in a small quantity of water, and gently squashed with a glass rod to ensure thorough wetting, spermatozoids emerge from the antheridia. After about 20 min. the suspension of spermatozoids may be removed with a pipette.

A cleaner and/or more concentrated sperm suspension can be obtained by taking advantage of the chemotactic reactivity of the spermatozoids. A drop of sperm suspension is placed on a glass plate under a low-power microscope and small droplets of sodium L-malate solution are carefully injected into the drop of sperm suspension. After 3–5 min., the aggregations of spermatozoids in these droplets may be removed with a fine-tipped pipette. This procedure may be repeated to obtain specially clean sperm suspensions for dark-ground photomicrography.

The crude sperm suspension obtained from the prothalli is contaminated with substances which act as pH buffers and with traces of chemotactically active substances. When a low activity of malate is required in the sperm suspension, or when accurate pH control is necessary with minimum concentrations of buffer, the spermatozoids must be washed several times by centrifugation and resuspension in buffer. This procedure causes some damage to the spermatozoids, which must not be confused with the effects of low malate concentration.

DISSOCIATION OF MALIC ACID

The dissociation of malic acid can be represented by

$$H_2M \rightleftharpoons HM^- + H^+ \qquad pK_1 = 3{\cdot}41,$$
$$HM^- \rightleftharpoons M^{2-} + H^+ \qquad pK_2 = 5{\cdot}05.$$

The relative concentrations of malic acid, H_2M, bimalate ion, HM^-, and malate ion, M^{2-}, existing at any pH can be calculated and are shown by the curves in Fig. 1.

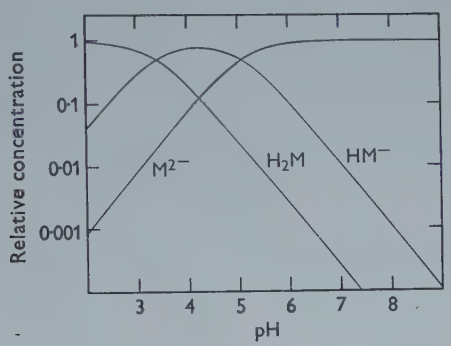

Fig. 1. Relative concentrations of malate species (logarithmic scale) at various pH's.

There are two forms of bimalate ion, depending on which carboxyl group is ionized. Consideration of the pK's of α- and β-hydroxy-butyric acids (3·9 and 4·5) indicates that bimalate will consist of about 80% of the form shown in Fig. 2*a* and about 20% of the form shown in Fig. 2*b*. In either form, the possibility of hydrogen bonding between the dissociated and undissociated carboxyl groups will tend to favour the *cis*-configuration. A hybrid structure (Fig. 2*c*) may best represent the bimalate ion, although there is no evidence that it is appreciably stabilized. On the other hand, where the two carboxyl groups are both neutral, as in the malic acid molecule (Fig. 2*d*), or both negatively charged, as in the malate ion (Fig. 2*e*), the *trans*-configuration will be strongly favoured, so that the two groups will be separated from each other as much as possible.

RESULTS

A drop of 0·1 M hydrochloric acid was placed on a glass slide in contact with a drop of sperm suspension containing 0·005 M *tris*-(hydroxymethyl) aminomethane—hydrochloric acid buffer, pH 8·1; 10^{-4} M sodium L-malate; and a small amount of pH indicator. Under these conditions the acid diffuses into the sperm suspension, neutralizes the buffer, and changes the colour of the pH indicator. A sharp colour change and, therefore, a sharp pH change occur at the junction of the region in which the buffer is completely neutralized with that in which it is not yet neutralized. This junction moves slowly across the drop of sperm suspension as the acid diffuses into the drop. A narrow aggregation of spermatozoids forms in this region of sharp

pH change (Brokaw, 1957). Dark-ground track photographs of the spermatozoids in this aggregation show them swimming actively within a narrow band about 75 μ wide and turning sharply back into the band from both sides.

The same phenomenon was observed with sperm suspensions buffered with 0·01 M sodium citrate, sodium phosphate, or sodium borate buffers, at pH's ranging from 5·6 to 8·7. With borate buffer, pH 8·7, and either cresol red indicator (pK 8·3) or brom cresol purple indicator (pK 6·3), the band of spermatozoids was located in

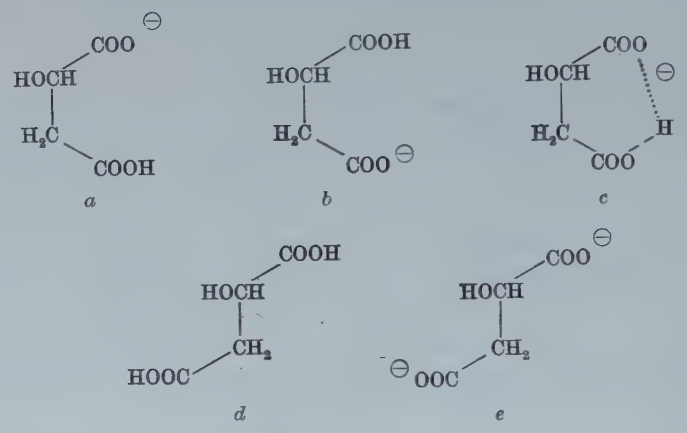

Fig. 2. Configurations of malate species. For details see text.

the same position as the colour change of the indicator, which confirmed the existence of a steep pH gradient in the region of sperm aggregation. With methyl-orange indicator (pK 3·5), the colour change was located slightly on the acid side of the band of spermatozoids. These experiments locate the region 'preferred' by bracken spermatozoids between pH 3·5 and 5·6.

When the spermatozoids are repeatedly washed by centrifugation and re-suspended in buffer solution without malate, no aggregation occurs when a pH gradient is established as described above. If 10^{-5} M sodium L-malate is added to a repeatedly washed sperm suspension, the spermatozoids again aggregate in a band when a pH gradient is established.

Fig. 1 shows that when a pH gradient extending from pH 2 to pH 8 is established in a solution of uniform malate concentration, there is a maximum concentration of the bimalate ion at pH 4·2 and bimalate gradients on either side of this point. The chemotactic response of bracken spermatozoids to these bimalate gradients could cause them to aggregate around pH 4·2, and explain the results of the above experiments.

It has not been possible to calculate precisely the pH gradient obtained when hydrochloric acid diffuses into a dilute buffer solution, as in the above situation. A very rough estimate of the resulting bimalate gradients indicates that they are sufficient to cause the observed aggregation, but this interpretation must be tentative until a more exact calculation of the gradients is achieved.

An alternative suggestion, which is more complex yet consistent with these observations, is that sensitivity to the undissociated malic acid molecule, plus an independent negative chemotaxis to low pH's, might produce this aggregation. Although there is some indication of negative chemotaxis to low pH's in washed sperm suspensions, the phenomenon of electrochemical orientation of bracken spermatozoids (Brokaw, 1957) suggests that the spermatozoids are sensitive to a charged ion.

Chemotaxis of bracken spermatozoids from a drop containing 10^{-4} M sodium L-malate and 0·015 M sodium phosphate buffer, pH 7·5, into one containing 10^{-4} M sodium L-malate and 0·015 M sodium phosphate buffer, pH 6·2, has been observed. The response is comparable to that observed when a drop containing 10^{-3} M sodium L-malate is placed in contact with one containing 10^{-4} M sodium L-malate, both drops containing the same buffer.

The gradient of the logarithm of concentration is the important variable in fern sperm chemotaxis (Pfeffer, 1884). Since Fig. 1 shows that the gradient of the logarithm of the bimalate ion concentration will be nearly equal to the gradient of pH at pH's above 6, the bimalate hypothesis predicts that bracken spermatozoids should be as sensitive to a pH gradient as to a malate gradient. This agrees with the above result, as the gradients set up by the diffusion of sodium phosphates will be of the same order of magnitude as the gradients set up by the diffusion of sodium malate.

Bracken spermatozoids orientate to a voltage gradient only if the sperm suspension contains a chemotactically active compound (Brokaw, 1957). The amount of sodium L-malate which must be added to obtain this electrochemical orientation is less at pH 6·8 than at pH 8·1 by approximately an order of magnitude. Fig. 1 shows that the malate concentration required to give a given concentration of bimalate ions will be twenty times less at pH 6·8 than at pH 8·1. The hypothesis that bimalate ions are required to sensitize spermatozoids to a voltage gradient is, therefore, in approximate agreement with the above result.

If the sperm suspension contains sodium maleate instead of sodium L-malate, addition of a drop of hydrochloric acid leads to the formation of a broader band of spermatozoids than when malate is used, and when the pH of the sperm suspension is low (6·2) there is no sharp aggregation. These observations suggest that in this case, too, the singly ionized ion, bimaleate, is the active species, and are consistent with the higher pK_2 of maleic acid (6·6).

DISCUSSION

Shibata (1911) suggested that malate might have a *cis*-configuration, since he, and Pfeffer, found maleate (*cis*) to be chemotactically active for many pteridophyte spermatozoids while fumarate (*trans*) was inactive. However, the malate ion, which predominates in solutions of pH above 6, is not likely to exist in the *cis*-configuration, while the bimalate ion will have a *cis*-configuration. Shibata also found that spermatozoids of *Equisetium arvense* L. and of *Salvinia natans* Allioni were positively chemotactic to solutions of dilute acids. He found that the sensitivity to a gradient of H^+ ions was just as strong as the sensitivity to a gradient of malate, and that the

effects of H^+ and malate concentrations were not additive. Since he did not show that the response to H^+ required the presence of malate, he concluded that the sensitivities to H^+ ions and to malate were independent. My experiments demonstrating the chemotactic interrelationship of malate and pH suggest that these spermatozoids are sensitive to the bimalate ion and not to the malate ion or the malic acid molecule, in agreement with all the above observations.

Shibata also studied the spermatozoids of *Isoetes japonica* A. Braun, which show positive chemotaxis to gradients of malate, fumarate, and OH^- ions. The above considerations suggest that in the case of *Isoetes* spermatozoids, the malate ion, rather than the bimalate ion, may be active. However, it is difficult to see how a pH gradient in solution above pH 6 could produce significant gradients of malate ion (see Fig. 1). It would be interesting to know if *Isoetes* spermatozoids are attracted by OH^- ions in the absence of malate.

SUMMARY

1. Bracken spermatozoids are chemotactically attracted by malic acid salts and in a pH gradient aggregate between pH 3·5 and 5·6.

2. This response to pH occurs only when the sperm suspension contains malate.

3. At higher pH's, the response to an H^+ gradient is as strong as the response to a malate gradient.

4. These observations suggest that bracken spermatozoids are sensitive to the bimalate ion rather than the malate ion. This is consistent with an earlier suggestion that chemotactic activity is associated with the *cis*-configuration, for the bimalate ion will have this configuration, while the malate ion and the malic acid molecule will have the *trans*-configuration.

This work has been carried out during the tenure of a National Science Foundation Predoctoral Fellowship. I am also indebted to Prof. G. Kenner for suggesting that the configurations of malate in solution might be significant, and to Lord Rothschild for introducing me to bracken sperm chemotaxis.

REFERENCES

Brokaw, C. J. (1957). 'Electro-chemical' orientation of bracken spermatozoids. *Nature, Lond.*, **179**, 525.

Pfeffer, W. (1884). Locomotorische Richtungsbewegungen durch chemische Reize. *Unter. Bot. Inst. Tübingen*, **1**, 364–482.

Rothschild, Lord (1956). *Fertilization*. London: Methuen.

Shibata, K. (1911). Untersuchungen über die Chemotaxis des Pteridophyten Spermatozoiden. *Jb. wiss. Bot.* **49**, 1–60.

CHEMOTAXIS OF BRACKEN SPERMATOZOIDS

IMPLICATIONS OF ELECTROCHEMICAL ORIENTATION

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(With Plate 7)

In the original experiments on the chemotaxis of fern spermatozoids, Pfeffer (1884) inserted small glass capillaries filled with a sodium malate solution into suspensions of spermatozoids, and observed that chemotactic aggregation resulted from precise orientation of the spermatozoids to the gradients produced by diffusion of malate from the open tip of the capillary. More recently, Rothschild (1952) provided definite confirmation of the precision of orientation in a chemical gradient by cinemicrographic records of chemotaxis, using bracken spermatozoids.

Pfeffer found that to obtain aggregation of spermatozoids at the tip of a capillary, there had to be a ratio of at least 30 between the original concentration of malate in the capillary and the concentration of malate in the sperm suspension. This 'difference threshold' was constant over a 100-fold range of concentrations. This indicated a relationship between the chemotactic response and the gradient of the logarithm of malate concentration, and Pfeffer emphasized the analogy between this relationship and the psychophysical principle known as Weber's Law.

The morphology of bracken spermatozoids corresponds closely to that of other fern spermatozoids described by Dracinschi (1930). The body contains a narrow nuclear strip, about 35 μ long, twisted into about three turns of a tapered spiral. Several dozen flagella, about 15 μ long, emerge from a strip of cytoplasm lying along the anterior half of the nucleus. A spherical cytoplasmic vesicle, about 10 μ in diameter, is attached to the posterior coil of the body.

The flagella propel the spermatozoid forward at speeds which may reach 300 μ/sec. As it moves forward, it rolls over three to six times per second, in the sense of a left-handed screw. The result is a helical path resembling those made by sea-urchin spermatozoa (Gray, 1955). Frequent changes in the direction of the axis of the helical path occur more or less at random, but there are no abrupt reversing reactions like those which have been held responsible for the chemotaxis of some bacteria and protozoa (Jennings, 1906).

Bracken spermatozoids orientate themselves with respect to an electric field when in a solution containing sodium L-malate (Brokaw, 1957). Further study of this phenomenon, and the chemical gradients existing in chemotaxis experiments, has suggested a tentative answer to the primary question posed by precise chemotactic orientation. How does the information available in a chemical gradient provide a directional stimulus to the organism, enabling it to turn and swim up the gradient?

CHEMICAL GRADIENTS

To study chemotaxis, known gradients of a chemotactically active substance must be established in a sperm suspension. This is done most conveniently by allowing the substance to diffuse into the sperm suspension from a region of higher concentration. The gradients must be calculated from an appropriate solution of the partial differential equation for diffusion, as no satisfactory method for direct measurement of the gradients within the suspension is available.

Pfeffer's capillary technique, as modified by Rothschild (1956) by using agar in the capillary to prevent hydrodynamic flow, thus ensuring that entrance of the active substance into the solution takes place entirely by diffusion, is experimentally convenient. The capillary can be introduced at a precise time with relatively little disturbance of the sperm suspension. Diffusion from the end of a cylindrical region into a thin layer of sperm suspension between slide and cover-glass must be considered. The solution of the diffusion equation for these boundary conditions has not been available in the past, but Prof. D. R. Hartree kindly examined this problem for me and obtained a solution to a somewhat idealized version. This shows that the concentration of diffusing substance will be nearly uniform throughout the depth of the suspension at distances from the tip of the capillary greater than the depth of the suspension, and has been used in the following form:

$$C = C_0 + (C_1 - C_0)\,(a^2/8b)\,(\pi Dt)^{-\frac{1}{2}} \exp(-\tfrac{1}{2}w^2)\,K_0(\tfrac{1}{2}w^2), \qquad (1)$$

where C = concentration of diffusing substance at a distance r from the tip of the capillary at a time t after the start of diffusion, C_0 = initial concentration in the suspension, C_1 = initial concentration in the capillary, $w = \frac{1}{2}r(Dt)^{-\frac{1}{2}}$, D = diffusion constant, a = internal radius of the capillary, b = half the thickness of the layer of sperm suspension, and K_0 = the modified Bessel function of the second kind, of zero order.

Before this solution was available, some work was done using capillaries filled with malate solution in agar, which had been rinsed for a known time, T, in distilled water immediately before insertion into the sperm suspension. The rate of diffusion from the capillary in the first few seconds after insertion was assumed to be relatively constant, and determined by the gradients established within the capillary during the period of rinsing. Diffusion in the suspension was then found using the solution for a continuous line source (Carslaw & Jaeger, 1947)

$$C = C_0 - C_1\,(a^2/8b)\,(\pi DT)^{-\frac{1}{2}} Ei(-w^2), \qquad (2)$$

where Ei is the exponential integral, defined by $Ei(x) = \int_{-\infty}^{x} \frac{e^u}{u}\,du$. This solution cannot be used for $r < 2b$.

Linear diffusion would be preferable to the radial diffusion obtained with Pfeffer's technique, as the mathematics of diffusion and of the analysis of sperm response to the gradients is simpler. Attempts to establish accurate and reproducible linear diffusion on a microscopic scale were unsuccessful, but a crude approximation to linear diffusion was obtained by placing two drops of solution in contact with each

other on a glass slide and attempting to get a straight boundary between the two drops. The solution of the diffusion equation used for linear diffusion is

$$C = C_0 + \tfrac{1}{2}(C_1 - C_0)\,(1 - \text{erf}\, w), \tag{3}$$

where C_0 and C_1 are the initial concentrations in the two drops, r = distance from the boundary in the drop of initial concentration C_0, and erf is the error function defined by $\text{erf}\, x = 2/\sqrt{\pi} \int_0^x e^{-u^2}\, du$.

Tables of the mathematical functions appearing in these equations are available (Flügge, 1954).

In these experiments, neutral solutions of sodium L-malate were used to establish malate diffusion gradients. It has been assumed that malate diffuses with a constant diffusion coefficient, D. The value of D used in calculations, 7×10^{-6} cm.^{2}/sec., is the mean of the values available for succinic and tartaric acids (Washburn, 1929). As the exact rate of diffusion of malate ion will depend on the ionic composition of the solutions used and on the concentration of malate, only approximate results can be expected when any of these methods of establishing and calculating malate gradients are used.

METHODS

(*a*) *Preparation of sperm suspensions*

Suspensions of bracken spermatozoids (*Pteridium aquilinum* (L.) Kuhn) were obtained as described in the preceding paper.

In experiments in which spermatozoids were placed in a voltage gradient, the suspension was buffered with 0·005 M *tris* (hydroxymethyl)aminomethane—hydrochloric acid buffer, '*tris* buffer', pH 8·1, to obtain maximum buffer capacity with minimum conductivity and, therefore, minimum production of toxic substances at the electrodes. In other experiments, 0·005 or 0·01 M sodium phosphate buffer, pH 7·5, was used, as the spermatozoids survived longer in this medium. Addition of small amounts of sodium and potassium ions to the *tris* buffer solution did not lead to longer survival.

(*b*) *Photographic records*

The movements of bracken spermatozoids were photographed with dark-ground illumination and exposures lasting several seconds. This is the 'dark-ground track' method used by Gebauer (1930) to study the galvanotaxis of *Polytoma uvella*, by Rothschild & Swann (1949) to study the movements of sea-urchin spermatozoa, and by Harris (1953) to study the chemotaxis of granulocytes. The sperm suspension was contained in a haemocytometer slide, providing a uniform depth of suspension of 0·10 mm. A 3 in. objective which was able to resolve sperm tracks at any level in the suspension was used. Short gaps were made in the track records by briefly interrupting the light beam, to record the times at which experimental changes were made, the direction of sperm movement, or a time scale.

(*c*) *Electrodes*

Silver-silver chloride electrodes were used to establish a voltage gradient in the sperm suspension. Two parallel electrodes, 5 mm. long and usually 10 mm. apart, made from 0·25 mm. silver wire, were used in experiments in an open drop of sperm suspension. In other experiments, four electrodes 4 mm. long, made from 0·05 mm. silver wire, were arranged as sides of a square 5 mm. wide in the haemocytometer slide. A silver-chloride layer was electrolytically deposited on the surfaces of the silver-wire electrodes. A roughly linear relationship between current and voltage was observed with these electrodes: when calculating the voltage gradient to which the spermatozoids were exposed the fall in potential between the electrodes was assumed to be linear. The effects of cell polarization, which might occur at the higher voltages used, and of the presence of the second pair of electrodes, parallel to the voltage gradients, were not determined.

The square array of electrodes was connected to a switching circuit so that a predetermined voltage gradient could be suddenly applied in any one of four directions. By this means, an individual spermatozoid could be kept in the microscope field, so that several responses to different gradients could be photographed.

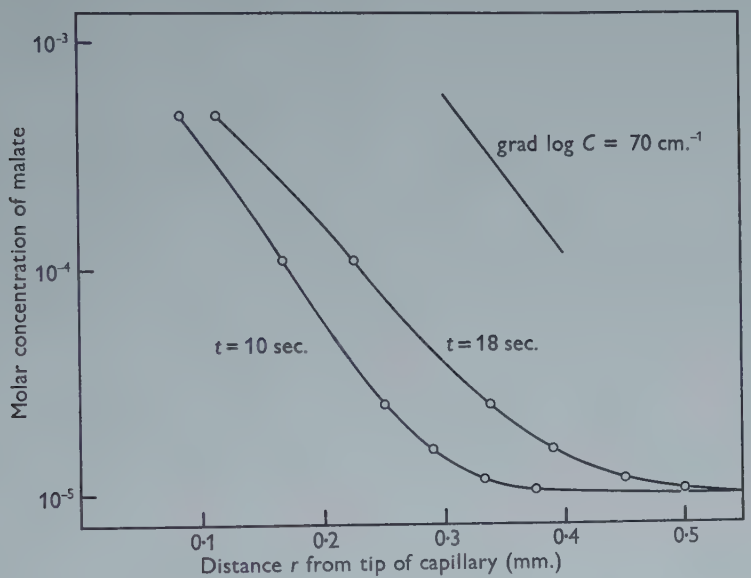
RESULTS

(*a*) *Chemotaxis*

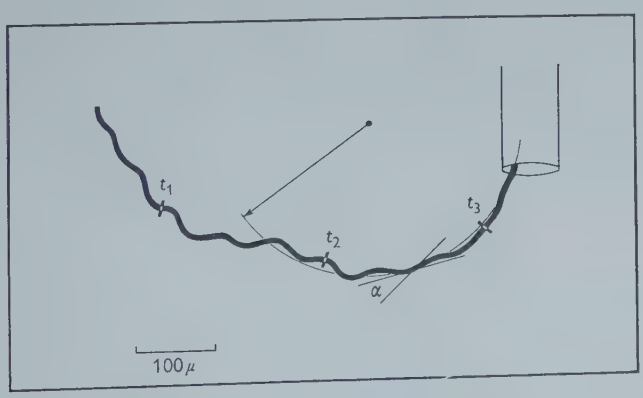
Many tracks of bracken spermatozoids swimming in a diffusion gradient near the tip of a capillary containing sodium L-malate in agar were recorded. Some of these are shown in Pl. 7, figs. 1–3. Pl. 7, figs. 1, 2, are mainly of qualitative interest, as no information about the malate gradients in these experiments was available. These tracks show that a gradual turning towards the tip of the capillary is superimposed on an apparently unaltered normal forward movement. In the experiment depicted in Pl. 7, fig. 3, the capillary was rinsed in water before insertion into the sperm suspension and the diffusion of malate was calculated from equation (2). The exposure started 10 sec. after the beginning of diffusion and lasted 8 sec. The malate concentration curves at 10 and 18 sec. are shown in Text-fig. 1. These curves are drawn on a logarithmic scale, following Pfeffer's demonstration of the importance of the relative concentration gradient, grad log C. Although, as discussed in the preceding paper, the spermatozoids are sensitive to the bimalate ion, this distinction is experimentally unimportant at constant pH, where the gradient of the logarithm of bimalate concentration will also equal grad log C.

Text-fig. 1 shows that near the capillary tip there is a region where grad log C is nearly constant. Calculation of grad log C is most accurate in this region. To determine the relationship between turning and the gradient, tracks such as that in Pl. 7, fig. 2, where all the turning takes place in the region of nearly constant grad log C, are of special interest. These are seldom found, as the weaker, less accurately known peripheral gradients generally influence the motion of the spermatozoids, before they enter the region of more constant grad log C. This can be seen in

Pl. 7, fig. 3, where two of the three spermatozoids which reached the tip were travelling in approximately the right direction before they entered the region where their motion was obviously influenced by the gradient. Most records were of this type. However, in Pl. 7, fig. 3, the third spermatozoid reaching the tip, whose track has a smoother curvature, allows a very rough calculation of the relationship between turning rate and grad log C, Text-fig. 2. Assuming that the turning rate



Text-fig. 1. Concentration of malate (logarithmic scale) for times at the start and finish of the exposure in Pl. 7, fig. 3, calculated by equation (2), with $T=571$ sec., $a=19\,\mu$, $C_1=0{\cdot}5$ M, $C_0=10^{-5}$ M.



Text-fig. 2. Calculation of turning rate for a sperm track from Pl. 7, fig. 3. t_1, t_2, t_3, indicate 0·86 sec. intervals; the average swimming speed is therefore about 250 μ/sec. The curved track is approximately an arc of radius 200 μ, corresponding to a turning rate of 1·25 rad./sec. From Text-fig. 1, grad log C is about 70/cm., and the angle α between the gradient and the direction of motion of the spermatozoid, is relatively constant and about 30°. The average turning rate per unit gradient is therefore 0·035 rad. sec.$^{-1}$/cm.$^{-1}$.

is proportional to the component of grad log C perpendicular to the direction of motion, the factor of proportionality is

$$\frac{\text{turning rate}}{\text{component of grad log } C} = 0{\cdot}035 \text{ rad. sec.}^{-1}/\text{cm.}^{-1}. \quad (4)$$

No other tracks which could be treated in this manner were obtained, so that it has not been possible to test the assumption of proportionality for chemotactic orientation. This isolated result, when the spermatozoids show so much variation in behaviour, is, of course, of limited value.

The photographic records of chemotaxis and the malate diffusion curves calculated from equations (1) and (2) show that unmistakable chemotaxis is observed when grad log C is greater than 25/cm., and that no chemotaxis is detectable when grad log C is less than 10/cm. In other experiments, the number of spermatozoids aggregating at the tip of the capillary was counted. When C_1/C_0 is high, a rapid aggregation of spermatozoids occurs in the first 2–3 min.; this is followed by a sharp levelling off, and after 5–8 min., by a slow decline in the number of spermatozoids near the tip. At 6 min., grad log C calculated from equation (1) for the region near the tip is 10/cm. A value of grad log C greater than about 10/cm. is therefore required to observe chemotactic aggregation with Pfeffer's technique.

Observation of a 'difference threshold' for chemotaxis with the capillary technique is not inconsistent with the assumption of a linear relationship between turning rate and grad log C. To observe chemotactic aggregation at the tip of a capillary, spermatozoids must be 'trapped' by the diffusion gradient. The turning rate of a spermatozoid entering the region around the capillary tip must be great enough for the spermatozoid to complete its turn towards the tip before it swims out of this region (Pl. 7, fig. 2). The path of a spermatozoid near a capillary will depend on its swimming speed, its random approach to the region around the tip, its turning rate in a gradient, and the magnitude of the gradient. As information about all these factors is available, it should be possible to calculate under what conditions chemotactic aggregation will be observed; but the mathematics of this problem are formidable. However, inspection of the diffusion curves calculated from equation (1) suggests that Pfeffer's threshold value for C_1/C_0, 30, is consistent with my measurements of the relationship between turning rate and grad log C.

(*b*) *Electrochemical orientation*

When a voltage gradient of more than 0·5 V./cm. is established in a sperm suspension containing *tris* buffer, pH 8·1, and 10^{-4}M sodium L-malate, the spermatozoids orientate and swim towards the anode. The voltage required is not noticeably altered by lowering the pH to 6·4 (sodium phosphate buffer, 0·01 M), by a tenfold increase in the ionic strength of the suspension, nor by increasing the concentration of sodium L-malate to 10^{-3}M. When the concentration of sodium L-malate in the suspension is decreased, a higher voltage must be applied to cause orientation of the spermatozoids. With 10^{-6}M malate at pH 8·1, no orientation is

observed at 7 V./cm.; the effect of higher voltage gradients was not investigated because of the toxic effects of the electrolytic products arising at the electrodes.

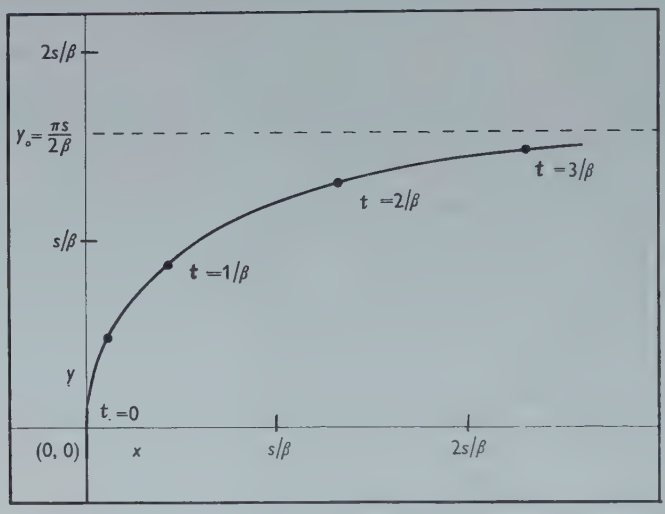
Sperm orientation occurs at 7 V./cm. with 10^{-4}M sodium L-malate or 10^{-4}M sodium maleate in the suspension, but not with 10^{-4}M sodium fumarate or 10^{-4}M sodium succinate (Brokaw, 1957). The chemical specificity of this phenomenon is, therefore, identical with that observed in classical chemotaxis experiments (Rothschild, 1956).

Voltage gradients in a sperm suspension can be manipulated more easily than chemical gradients, and therefore provide a more favourable situation for quantitative study of the response of spermatozoids to a gradient. Pl. 7, fig. 4, shows the response of several spermatozoids to a high voltage gradient applied at right angles to the direction of swimming. The spermatozoids were initially swimming towards the top of the plate under the influence of a voltage gradient in that direction. At the time indicated by the first gap in the tracks, this gradient was removed, which produced no response. At the time indicated by the second gap in the tracks, a horizontal gradient was applied. This experiment shows that the spermatozoids can turn very rapidly and also that there is much individual variability in their movements. The endosmosis of particles in the suspending medium, also seen in this photograph, indicates the exact direction of the voltage gradients. The turns do not show the consistent dependence on orientation within the path-helix indicative of a single-unit klinotactic mechanism, as in the photo-orientation of *Euglena* (Mast, 1938).

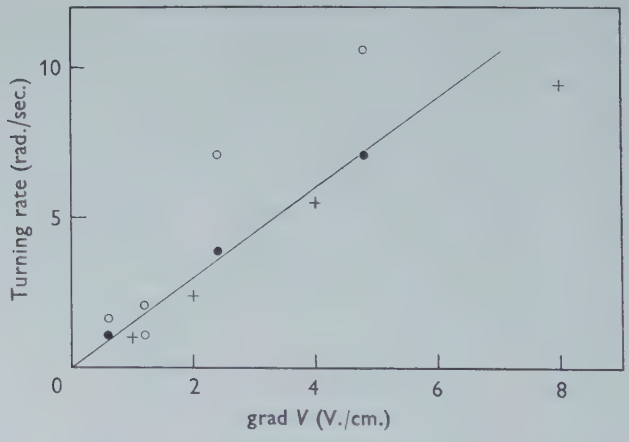
Responses to lower voltage gradients are shown in Pl. 7, figs. 5, 6. In Pl. 7, fig. 5, a spermatozoid was initially swimming towards the top of the plate from the lower left-hand corner. A horizontal gradient of 1 V./cm. was then applied, causing a slow turn to the right. A gradient of 2 V./cm. was then applied in the opposite direction, causing a turn of 180°. The turning rate is higher at the higher voltage and highest when the spermatozoid is swimming at right angles to the gradient. This suggests a direct proportionality between turning rate and the component of the voltage gradient perpendicular to the axis of the spermatozoid's helical path. If this is so, the expected shape of the curved track can be calculated (see Appendix 1) and is shown in Text-fig. 3. This is similar to the curves in the track in Pl. 7, fig. 5. Most tracks do not have exactly the form shown in Text-fig. 3, as the normal helical motion of the spermatozoid obscures the form of rapid turns at high voltage gradients; while at lower voltage gradients, the probability is high of a spermatozoid making random turns before completing orientation to the gradient. However, turns resembling that drawn in Text-fig. 3 are observed often enough to suggest that this is an accurate representation of the turning of the spermatozoids.

The relationship between turning rate and voltage gradient was also investigated by photographing a series of responses of an individual spermatozoid to various voltage gradients. Part of one series of results is shown in Pl. 7, fig. 6. This spermatozoid was initially swimming towards the top of the plate, on the left-hand side. A horizontal voltage gradient of 4·8 V./cm. was applied, followed by a vertical gradient of 0·6 V./cm., and then a horizontal gradient of 2·4 V./cm. The

higher voltage gradients caused sharper turns. Assuming that these turns have the shape shown in Text-fig. 3, the distance y_0 can be measured, and one can compute β, the turning rate when the spermatozoid is swimming at right angles to the gradient. Data for eight turns made by the spermatozoid in Pl. 7, fig. 6, and



Text-fig. 3. Predicted path of a spermatozoid orientating to a gradient applied in the x direction at $t=0$. At $t=0$, the spermatozoid is at (0, 0), travelling in the y direction with speed s (see Appendix 1).



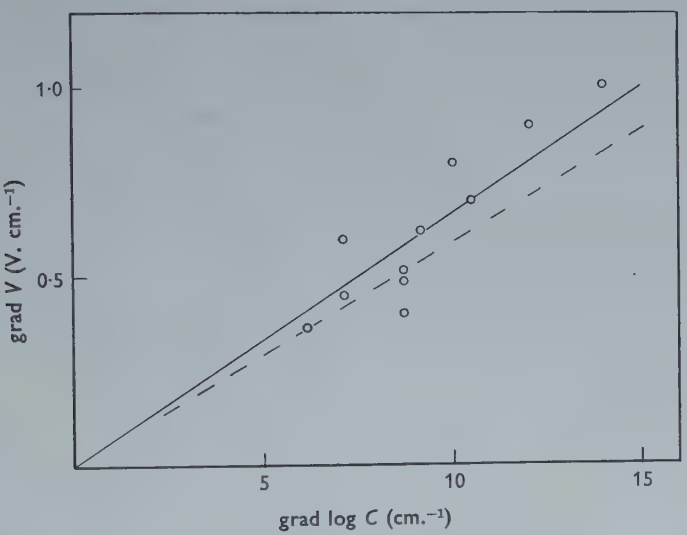
Text-fig. 4. Turning rates of spermatozoids reacting to voltage gradients. ●=turns shown in Pl. 7, fig. 4. ○=other turns of this spermatozoid. + =turns of another spermatozoid.

four turns made by another spermatozoid are shown in Text-fig. 4, where β is plotted against the applied voltage gradient, grad V. These results suggest that the assumption of a linear relationship between turning rate and the magnitude of the gradient is reasonable, and that, at least for these two spermatozoids,

$$\beta/\text{grad } V = 1{\cdot}5 \text{ rad. sec.}^{-1}/\text{V. cm.}^{-1}. \qquad (5)$$

(*c*) *Combination of chemical and electric gradients*

A direct determination of the quantitative relationship between the responses to chemical and electric gradients was effected as follows: linear diffusion of malate was established between two drops of solution placed in contact on a glass slide, and two electrodes were arranged parallel to the boundary between the drops. The voltage across the electrodes was varied and the voltage which just neutralized the effect of the concentration gradient, so that the spermatozoids swam in random directions, was noted. The precision of the results is limited by the difficulty of establishing exact linear diffusion and of determining when the gradients are



Text-fig. 5. Voltage gradient required to neutralize chemical gradient. Solid line represents mean of observed points, dashed line represents the ratio predicted by equation (9).

balanced. Errors may also be introduced if the voltage gradient is not linear, due to variation in the depth of the drops. The diffusion junction potential and the effect of the applied voltage gradient on the diffusion of malate have been neglected, which may introduce second-order errors.

The drop of sperm suspension contained 2×10^{-4}M sodium L-malate and 0·005M *tris* buffer, pH 8·1, and the other drop contained 2×10^{-3}M sodium L-malate and 0·005M *tris* buffer. Debris in the sperm suspension indicated the boundary between the two drops and only those experiments in which a fairly straight boundary was obtained were continued.

In this linear diffusion situation there will be a fairly broad region on the low-concentration side of the boundary between the drops, where grad log C is roughly constant near its maximum value. In these experiments, the applied voltage was raised until the spermatozoids began to swim across the region of maximum grad log C into the drop of lower malate concentration; the voltage was then lowered

until the spermatozoids began to swim in the opposite direction; the mean of these two voltages was recorded together with the time elapsed after joining the two drops. The results from four successful experiments are shown in Text-fig. 5, where the applied voltage gradients (grad V) are plotted against grad log C calculated by equation (3) for the region of maximum grad log C at the time of the determination. These results provide some support for the assumption of a linear relationship between responses to chemical and electric gradients; the factor of proportionality between the two gradients for equal effects, grad log C/grad V, is about 15.

The same result was obtained when the experiment was repeated with malate concentrations of 2×10^{-5} and 2×10^{-4} M in the two drops.

This result is compared with two other estimates of the factor of proportionality between the gradients in Table 1.

Table 1. *Summary of data on the equivalence between the effects of chemical and electric gradients*

Experiment	Result in electric gradient	Result in chemical gradient	$\frac{\text{grad log } C}{\text{grad } V}$
Measurement of minimum gradient required for observation of tactic response	0·5 V. cm.$^{-1}$	10 cm.$^{-1}$	20
Measurement of rate of turning of individual spermatozoids in a gradient	1·5 rad. sec.$^{-1}$/V. cm.$^{-1}$	0·035 rad. sec.$^{-1}$/cm.$^{-1}$	42
Direct balancing of voltage gradient and diffusion gradient	—	—	15

DISCUSSION

These results indicate that there is a close relationship between chemotaxis and electrochemical orientation. When a spermatozoid is in a solution containing bimalate ions, a voltage gradient produces an effect which the sensory elements of the spermatozoid cannot distinguish from a concentration gradient of bimalate ions.

One effect common to these two situations is a net flux of bimalate ions. The velocity of a univalent anion exposed to a concentration gradient and a voltage gradient will be (Höber, 1950)

$$v = -2{\cdot}3D \text{ grad log } C + u \text{ grad } V, \tag{6}$$

where v = net velocity of the ion, D = its diffusion coefficient, and u = its mobility. Since, at 25° C.,

$$u/2{\cdot}3D = f/2{\cdot}3RT = 17, \tag{7}$$

where f = faraday, R = the gas constant, and T = absolute temperature, the movement of bimalate ions will be neutralized by an applied voltage gradient when

$$\text{grad log } C/\text{grad } V = 17. \tag{8}$$

However, to do this, the direction of the voltage gradient must be the reverse of that applied to prevent chemotaxis in the experiment described above. The spermatozoids cannot, therefore, be sensitive to the flux of bimalate ions. However, the numerical agreement between the experimentally determined ratios (Table 1) and that in equation (8), and the appearance of grad log C in these equations, suggests that some physicochemically similar sensory effect is involved.

A possible explanation is that the sensory elements of the spermatozoid adsorb bimalate ions. A body carrying reversibly adsorbed bimalate ions will, in a voltage gradient, experience a force tending to move it towards the anode. In a bimalate concentration gradient it will experience a force tending to move it up the gradient (see Appendix 2). Such forces could most simply cause an orientating response if bimalate ions were adsorbed on the anterior end of the spermatozoid, so that the forces would directly pull the anterior end around until the spermatozoid was swimming up the gradient.

This suggestion can be treated more exactly by considering a model consisting of a sphere of radius $\rho = 5\,\mu$. A small region on the surface of the sphere contains 'bimalate combining sites' which reversibly adsorb bimalate ions. The sphere moves at constant speed through the solution with the adsorbing region in front. Its tactic responses are the sum of this constant movement and the independent orientation of the sphere caused by forces acting on the adsorbing region.

If the effect of viscous drag is not considered, the force acting on the model when it is moving at right angles to a gradient will be (see Appendix 2)

$$\text{turning force} = N\,(2{\cdot}3RT \text{ grad log } C + f \text{ grad } V), \qquad (9)$$

where N = moles of adsorbed bimalate. The factor $f/2{\cdot}3RT$ also appears here, agreeing in both direction and magnitude with the observed relationship between the tactic effects of chemical and electric gradients (Table 1). Using equation (9) and Stoke's law for the frictional resistance of a rotating sphere, it can be estimated that about 10^5 bimalate ions would have to be adsorbed to give the model the turning rate given by equation (5).

However, when viscous effects are considered, the force predicted by equation (9) will not be attained, because when the sphere turns it will drag along with it the layer of solution near its surface. In a voltage gradient, the movement of the excess cations in this layer will produce a retarding force, and the resultant net movement must be calculated by the quite different approach used for electrophoresis (Abramson, Moyer & Gorin, 1942). When this is done, the estimate of the number of bimalate ions neededto turn the model is increased to 10^7–10^8. 10^8 ions is probably close to an upper limit for the number of bimalate ions which could be adsorbed on the anterior surface of a spermatozoid.

In a concentration gradient, the movement of the surface layer of the solution along with the sphere means that the adsorbing surface is not really moving relative to the gradient, as implied in the derivation of equation (9), and the force will therefore be less. Since both these effects are due to the movement of the surface layer of solution, they may, perhaps, be of similar magnitude, so that equation (9) will still

explain the experimental results. Further information about the physical chemistry of an adsorbing body in a gradient is needed before it can be concluded that the behaviour of this model will duplicate the tactic behaviour of bracken spermatozoids.

Previous students of the chemotaxis of fern spermatozoids have held that turning is a result of alterations in the activity of the flagella (Hoyt, 1910; Metzner, 1926). However, no mechanism describing the relationship between flagellar activity and malate concentration has been offered which is consistent with all the following observations.

(1) No observable change in swimming speed or any other parameter of general flagellar activity is found when a spermatozoid is turning and moving up a gradient (see Pl. 7, figs. 1, 2).

(2) There is a linear relationship between turning rate and grad log C or grad V, without a measurable threshold.

(3) There is no delay, at least in responding to a voltage gradient (see Pl. 7, fig. 4).

(4) The sensitivity to a voltage gradient requires bimalate or other chemotactically active ions.

The hypothesis suggested here, in which turning is independent of flagellar activity, satisfies all the above conditions, and is also consistent with the following observations.

There is no evidence that malate is metabolized by bracken spermatozoids.

The ratio between the gradients required for equal tactic effects is in agreement with equation (9): subject to further information about the validity of equation (9).

SUMMARY

1. The chemotaxis of bracken spermatozoids involves their precise orientation in a gradient of bimalate or a few other similar ions. When a voltage gradient is established in a sperm suspension containing bimalate or other chemotactically active ions, a similar orientation is observed, causing the spermatozoids to swim towards the anode.

2. Photographic records of sperm responses reveal a linear relationship between turning rate and the component of the gradient perpendicular to the direction of swimming.

3. Estimates have been obtained of the ratio of the magnitudes of the two types of gradient required to produce an equal tactic response.

4. The results suggest that the sensory elements of the spermatozoids adsorb bimalate ions. The reversible adsorption of bimalate ions on 'bimalate-combining sites' on the anterior end of a spermatozoid might fully explain its tactic behaviour, without requiring any modification of flagellar activity by bimalate.

The author wishes to acknowledge his indebtedness to the National Science Foundation for providing a predoctoral fellowship, to Prof. Sir James Gray for providing accommodation in his department, to Prof. H. C. Longuet-Higgins and

Dr L. Orgel of the Department of Theoretical Chemistry at Cambridge for several valuable discussions, and to Lord Rothschild for continued advice and encouragement.

APPENDIX 1

Calculation of the path of a particle moving in a plane at constant speed and turning at a rate determined by the angle between its direction of motion and a fixed direction

The basic assumptions are contained in the following differential equation:

$$d\theta/dt = -\beta \sin\theta, \tag{i}$$

where θ = angle between the direction of motion of the particle and a fixed direction, t = time, and β = a constant, the turning rate when $\theta = \frac{1}{2}\pi$. If the fixed direction (the direction of the gradient) is along the positive x axis, and the particle is at (0, 0) at $t = 0$, moving along the y axis with speed s, the equations

$$\left.\begin{aligned} \tan\tfrac{1}{2}\theta &= \exp -\beta t, \\ y &= s\int_0^t \sin\theta\, dt, \\ x &= s\int_0^t \cos\theta\, dt, \end{aligned}\right\} \tag{ii}$$

determine the curve. Integrating and eliminating θ,

$$\left.\begin{aligned} y &= s/\beta\,(\tfrac{1}{2}\pi - 2\tan^{-1}(\exp -\beta t)), \\ x &= st - s/\beta \ln \tfrac{1}{2}(1+\exp -2\beta t). \end{aligned}\right\} \tag{iii}$$

The curve in Fig. 3 can be drawn from (iii). For large t,

$$y_0 \equiv \lim_{t\to\infty} y = \pi s/2\beta. \tag{iv}$$

y_0 can conveniently be measured to determine β.

APPENDIX 2

Prediction of the behaviour of an adsorbing surface in a concentration gradient

Consider first the case of a cylindrical surface of circumference c, extending through the interface between two immiscible liquids, A and B (Text-fig. 6*a*). If γ_A is the interfacial tension between liquid A and the surface of the cylinder and γ_B is that between liquid B and the surface, a force $-F$ will be required to hold the system in equilibrium. When the cylinder is allowed to move a distance δx through the interface against this force at equilibrium, the work done will be

$$\delta W = -F\,\delta x. \tag{v}$$

The interfacial tension of the surface element moved through the interface will change from γ_B to γ_A, corresponding to a change in surface energy

$$\delta E = c\,\delta x\,(\gamma_A - \gamma_B). \tag{vi}$$

Since, for the process at equilibrium,

$$\delta E + \delta W = 0, \tag{vii}$$

$$-F = c(\gamma_B - \gamma_A). \tag{viii}$$

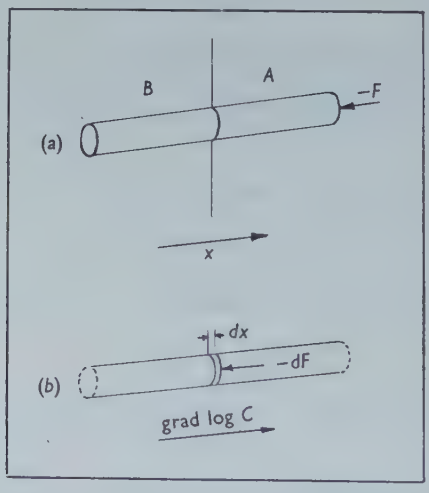
$-F$ will be positive if $\gamma_B > \gamma_A$, so that the cylinder will tend to move through the interface into the liquid of lower interfacial tension. This has been qualitatively demonstrated (Freundlich, 1926) and the quantitative relationships are straightforward.

Consider now that A and B are two solutions containing a solute which is adsorbed on the surface. The interface will be replaced by a stiff membrane which fits closely around the cylinder. The interfacial tension between the surface and the solutions will depend on the concentration of solute, according to the Gibb's adsorption equation

$$\Gamma_A = -\partial\gamma_A/\partial\mu_A, \tag{ix}$$

where μ_A = chemical potential of the solute in A, and Γ_A = excess number of moles of solute per unit area adsorbed on the surface in A. If the solute concentrations in A and B are not too different, the force required to hold the cylinder in equilibrium will be, in the same way,

$$-F = c(-\Gamma_B\mu_B + \Gamma_A\mu_A). \tag{x}$$



Text-fig. 6. Forces on adsorbing cylinder.

If the surface of the cylinder is nearly saturated with an adsorbed solute, Γ will not vary much with concentration, and

$$-F = c\Gamma(\mu_A - \mu_B). \tag{xi}$$

Since

$$\mu_A = \mu_A^* + 2{\cdot}3RT \log C_A, \tag{xii}$$

where μ_A^* is a constant,

$$-F = c\Gamma 2{\cdot}3RT\,(\log C_A - \log C_B). \tag{xiii}$$

The cylinder will, therefore, tend to move through the membrane into the solution of higher concentration, since the higher concentration lowers the interfacial tension.

If the membrane is removed, a gradient of solute concentration will be established but the same force should be available. If a small element of surface of the cylinder $c\,dx$, is moved a distance $\delta x (dx \ll \delta x \ll 1)$ along a gradient at equilibrium (Text-fig. 6*b*), the change in surface energy will be

$$\delta E = (c\,dx)\,\delta\gamma = -(c\,dx)\,\Gamma 2{\cdot}3RT \text{ grad log } C\,\delta x, \qquad \text{(xiv)}$$

and the force, $-dF$, to hold this surface element at equilibrium will be

$$-dF = c\Gamma 2{\cdot}3RT \text{ grad log } C\,dx. \qquad \text{(xv)}$$

If the adsorbing surface has total area A, with grad log C constant over the surface, and

$$N \equiv \Gamma A, \qquad \text{(xvi)}$$

the total force tangential to the adsorbing surface required to prevent the surface from moving up the gradient will be

$$-F = N2{\cdot}3RT \text{ grad log } C. \qquad \text{(xvii)}$$

This equation will not hold for a surface of arbitrary shape, but will be satisfactory for a small element of the surface of a sphere, as needed for equation (9).

When the membrane is removed, the surface layers of the solution will no longer be prevented from moving along with the surface, and the surface will not actually move relative to the gradient, as stated above. No calculation of the magnitude of this effect is available and no experimental work on this problem is known.

REFERENCES

Abramson, H. A., Moyer, L. S. & Gorin, M. H. (1942). *Electrophoresis of Proteins.* New York: Reinhold.

Brokaw, C. J. (1957). 'Electro-chemical' orientation of bracken spermatozoids. *Nature, Lond.*, **179**, 525.

Carslaw, H. S. & Jaeger, J. C. (1947). *Conduction of Heat in Solids.* Oxford: Clarendon Press.

Dracinschi, M. (1930). Über das reife spermium der Filicales. *Ber. Dtsch. Ges.* **48**, 295–311.

Flügge, W. (1954). *Four-place Tables of Transcendental Functions.* London: Pergamon Press.

Freundlich, H. (1926). Hatfield, H. S., trans. *Colloid and Capillary Chemistry.* London: Methuen.

Gebauer, H. (1930). Zur Kenntnis der Galvanotaxis von Polytoma uvella und einigen anderen Volvocineen. *Beitr. Biol. Pfl.* **18**, 463–500.

Gray, J. (1955). The movement of sea-urchin spermatozoa. *J. Exp. Biol.* **32**, 775–801.

Harris, H. (1953). Chemotaxis of granulocytes. *J. Path. Bact.* **66**, 135–46.

Höber, R. (1950). *Physical Chemistry of Cells and Tissues.* Philadelphia: Blakiston.

Hoyt, D. (1910). Physiological aspects of hybridization and fertilization in ferns. *Bot. Gaz.* **49**, 340–70.

Jennings, H. S. (1906). *Behaviour of the Lower Organisms.* New York: Columbia University Press.

Mast, O. (1938). Factors involved in the process of orientation of lower organisms to light. *Biol. Rev.* **13**, 186–224.

Metzner, P. (1926). Studien über die Bewegungsmechanik der Spermatozoiden. *Beitr. allg. Bot.* **2**, 433–97.

Pfeffer, W. (1884). Locomotorische Richtungsbewegungen durch chemische Reize. *Unter. Bot. Inst. Tübingen* **1**, 304–82.

Rothschild, Lord (1952). *Int. Rev. Cytol.* **1**, 257–63.

Rothschild, Lord (1956). *Fertilization.* London: Methuen.

Rothschild, Lord & Swann, M. M. (1949). The fertilization reaction in the sea-urchin egg. *J. Exp., Biol.* **26**, 164–76.

Washburn, E. W., ed. (1929). *International Critical Tables*, Ed. 1, **5**, 70.

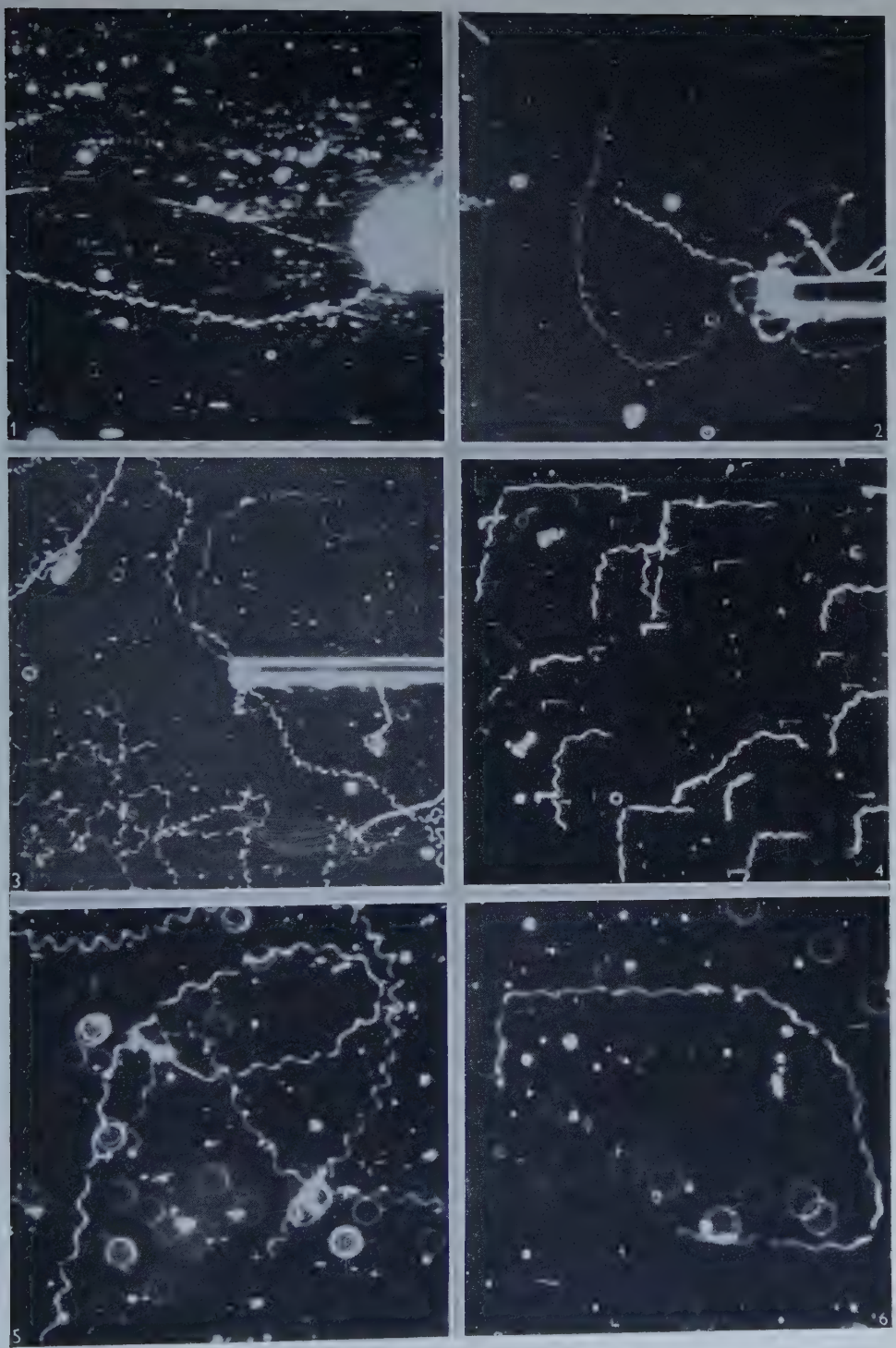
EXPLANATION OF PLATE 7

Figs. 1, 2. Examples of sperm tracks approaching tip of a capillary source of sodium L-malate. Time intervals of $\frac{1}{2}$ sec. are indicated by gaps in the tracks. Direction of swimming, towards tip, was determined by observation during exposure. ×60.

Fig. 3. Tracks of three spermatozoids approaching the tip of a rinsed capillary source of sodium L-malate, 10–18 sec. after the start of diffusion. Gaps in the tracks producing an asymmetric sequence of increasing lengths indicate the direction of swimming and the time scale. Time for one cycle of the sequence = 0·86 sec. ×45.

Fig. 4. Tracks of spermatozoids reacting to a strong voltage gradient. Gaps in the tracks indicate changes in the applied gradient (see text). ×45.

Figs. 5, 6. Tracks showing the responses of individual spermatozoids to several changes in the voltage gradient (see text). Gaps in the tracks indicate time intervals of 1 sec. ×75.



BROKAW—CHEMOTAXIS OF BRACKEN SPERMATOZOIDS.

(*Facing p.* 212)

THE SURVIVAL OF *ARTEMIA SALINA* (L.) IN VARIOUS MEDIA

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INTRODUCTION

The range of chemical and physical conditions under which the cells and tissues of Metazoa can function is limited. Yet some animals are capable of living in very odd and extreme environments.

Excellent examples of extreme environments are found in highly saline lakes and pools. These vary considerably in their total osmotic concentration, and in the nature of the solutes present. The ability of *Artemia salina* to live in such brines has long been known (for data on distribution see Daday, 1910; Abonyi, 1915; Stella, 1933; Kuenen, 1939; Barigozzi, 1946; etc.).

It is of interest to know the types of solutes that can be tolerated, particularly as claims have been made that *Artemia* can survive in several most unlikely media. For example, Boone & Baas Becking (1931) state apparently from hearsay: 'It has been known for a long time that *Artemia* is capable of living for days in solutions of potassium permanganate, potassium bichromate, and silver nitrate.' Such statements as these might be taken as evidence that *Artemia* is highly impermeable. A study of the survival of *Artemia* in various media may thus give information concerning the permeability of the animal and possibly also about the regulatory mechanisms.

Previous work on the survival of *Artemia* in various media has been done by Martin & Wilbur (1921) on the adults, and by Boone & Baas Becking (1931); Jacobi & Baas Becking (1933); Baas Becking, Karstens & Kanner (1936) on the hatching and survival of the nauplii. This previous work was mainly concerned with the effects of sodium, potassium, magnesium, and calcium ions, and, where this work overlaps with that presented below, the results are comparable. Corner & Sparrow (1956) have also studied the toxicity of copper and mercury compounds to *Artemia* larvae.

MATERIAL AND METHOD

The actual systematic position of *Artemia salina* is not clear. There appear to be numerous variable races. Daday (1910) regarded all the salt-water forms as belonging to one species *A. salina* (L.), but other authors have claimed that there are several distinct species. Cytogenetical work (e.g. Stella, 1933; Barigozzi, 1946; Goldschmidt, 1952) has shown a complex picture of different genetic races, and it is difficult to apply normal species criteria. In this and subsequent papers *Artemia* has been regarded as a single species, *Artemia salina* (L.).

Dried eggs obtained from the U.S.A. were used. The animals were reared in large aquaria containing gently aerated sea water. The temperature range of the aquaria was maintained to within about 19–24° C. A suspension made by grinding up dried yeast pellets in sea water was occasionally added as food. Self-reproducing cultures have been maintained in this way for several years.

The experiments were done on adults, which were about 10–12 mm. long, and at room temperature (18–19° C. usually). Batches of animals were transferred to various media, and the time until the animals had become moribund was observed. The animals were considered to be moribund when all limb movements had ceased or had become reduced to slow ineffective twitches. Considerable differences were seen between individuals and between experiments done at different times. The actual times quoted are therefore to be treated as giving only the approximate magnitude of survival time.

RESULTS

Sea water

Artemia survives indefinitely in sea water, which may be regarded as a balanced salt solution. After gradual acclimatization, numbers of individuals will survive indefinitely in various dilutions and concentrations of sea water, ranging from 10% sea water up to and including a saturated crystallizing sea-water brine. The majority of habitats where *Artemia* occurs probably have ionic ratios fairly similar to these sea-water brines. The absence of *Artemia* from the sea itself is presumably due to ecological rather than physiological factors.

Distilled water

The survival time of *Artemia* placed in glass distilled or tap water is relatively short, suggesting a fair degree of permeability. The swimming becomes very much slowed after about 12–18 hr., and most are dead or moribund by 24 hr.

Regarding distilled water as a neutral standard (i.e. non-toxic), we can say that those solutes that extend this survival time are favourable to the animal, and those that markedly reduce it are toxic.

Substances prolonging survival time

(1) *Sodium chloride.* *Artemia* adults survive for several days in pure NaCl solutions (0·5 M-NaCl, which is approximately isotonic with sea water, was used). This result would be expected as NaCl is the major constituent of most natural brines.

(2) *Sodium bromide.* Many individuals survived actively for well over 30 hr. in 0·5 M-NaBr solution.

(3) *Mannitol.* The survival time in a solution of mannitol approximately isotonic with the haemolymph was prolonged several hours over the distilled-water time. This is presumably due to the osmotic effect decreasing water flux through the animal, thus slowing loss of ions.

Substances with slight or slow toxicity

(1) *Certain sodium salts.* Sodium sulphate (0·25 M) allowed active survival for more than 12 hr., though animals were moribund or very slow by 24 hr. Sodium nitrate (M) allowed active survival for about 5–6 hr. Sodium benzene-sulphonate (0·5 M) allowed many to survive actively for about 8 hr.

Vinogradov (1953) states that *Artemia* is found in some Russian lakes where sulphates are present in high concentration, in some cases even forming a saturated solution of Na_2SO_4. As Na_2SO_4 by itself is scarcely toxic, there seems no reason why *Artemia* should not have adapted to these conditions, provided that chloride and other ions are also present.

(2) *Magnesium, calcium, lithium, and choline salts.* In a 0·25 M-magnesium chloride solution animals survived actively for 6–9 hr. In 0·25 M-calcium chloride toxic symptoms appeared a little faster. In 0·5 M-choline chloride the animals were moribund within 2–4 hr. In 0·5 M-lithium chloride the animals were moribund in 2–3 hr.

Rapidly toxic substances

These substances in concentrations similar to those mentioned above would cause death in minutes rather than in hours.

(1) *Dilute acids.* 5% nitric acid killed the animals in 2–3 min.

(2) *Sodium bicarbonate.* Very surprisingly in a M-$NaHCO_3$ solution there was loss of mobility and apparently death within 5 min. As the sodium ion is not toxic, it is probable that pH or narcotic effects due to the bicarbonate ion are responsible.

(3) *Metabolic inhibitors.* Animals placed in sea water plus 2% sodium azide were usually very moribund by 30 min. Animals placed in a 15% solution of ethyl urethane were very much slowed or moribund in 30–60 min.

(4) *Fixatives.* Alcoholic fixatives (e.g. Carnoy and alcoholic Bouin), absolute alcohol, and acetone are lethal within 1 min. Aqueous fixatives are less rapid. Death occurs within 10 min. in Susa and within about 15 min. in aqueous Bouin.

(5) *Silver salts.* Even dilute solutions of $AgNO_3$ were found to be very highly toxic. The animals were removed from sea water, rinsed in distilled water to remove adherent chloride, and then placed in dilute $AgNO_3$ solutions. In a 10 mM./l. solution the animals were moribund in about 5–10 min. Even in a 0·1 mM./l. solution most of the animals were moribund in 20 min. If both ends of the gut were ligatured with fine threads (made by teasing out bolting silk) to prevent swallowing, the response to $AgNO_3$ was not affected. There was no appreciable difference in the survival time as between 10 mM./l. $AgNO_3$, and M-$NaNO_3$ + 10 mM./l. $AgNO_3$ (Na:Ag ratio 100:1).

(6) *Potassium salts.* One of the most remarkable findings of earlier work was the high toxicity of potassium salts to *Artemia* (Martin & Wilbur, 1921; Boone & Baas Becking, 1931; Jacobi & Baas Becking, 1933). In the present work 0·5 M. solutions of potassium chloride, potassium nitrate, and potassium benzene-sulphonate have been used. In all these the animals become moribund within 30 min. A similar

rapid effect was seen when the animals were ligatured at head and anus to prevent swallowing.

A saturated solution of potassium permanganate kills *Artemia* within 15 min. But with this salt the permanganate ion would also be expected to have a toxic action.

Baas Becking and his associates (working on the hatching and survival of nauplii) claimed that the toxic effect of potassium ion (and also that of magnesium and calcium ions) could be antagonized by the presence of sodium ions. However, their experiments were done by mixing the chlorides of two and in some cases more cations in various proportions to give a constant total molarity, and repeating the experiment at a relatively small number of other total molarities. This makes it rather difficult to interpret their results, particularly as the antagonism ratios appeared to vary with changes in the total molarity.

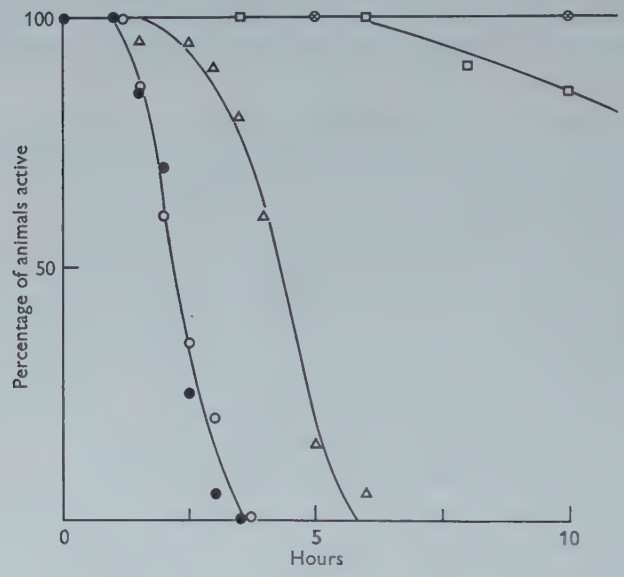

Fig. 1. The survival of *Artemia* in media with various Na:K ratios.

	Composition of media	
	K mM./l.	Na mM./l.
●	100	0
○	100	100
△	100	500
□	100	1000
⊗	100	1800

In the present work on adult *Artemia* a simpler procedure giving a clearer result was used. In these experiments the potassium concentration was not varied. A series of solutions was made up each containing 100 mM./l. KCl, and from 0 to 1800 mM./l. NaCl. Groups of adult animals were transferred from a sea-water culture to these various solutions. The survival time is presented graphically (Fig. 1). The results

clearly indicate a competitive or antagonistic effect between sodium and potassium ions, and that for prolonged survival the Na:K ratio must equal or exceed 10.

The ecological consequences of the potassium sensitivity are probably important, and *Artemia* is absent from desert lakes rich in potassium (Boone & Baas Becking, 1931).

DISCUSSION

It is clear that, although *Artemia* can live over a very wide concentration range, it is by no means insensitive to the chemical composition of its environment, and this indicates a degree of permeability. In fact the only media in which *Artemia* can survive for long periods are solutions in which certain sodium salts predominate. Many substances are extremely toxic. These include substances to which *Artemia* has in the past often been regarded as resistant. The speed with which certain toxic substances act suggests that they are entering through, or acting on, a permeable part of the external surface. Further evidence for this, in the case of silver and potassium ions, was obtained in the experiments where the gut was isolated from the medium by ligatures.

However, it is clear that *Artemia* is much less sensitive to the chemical nature of its environment than many other animals, as judged by the concentration range over which the animal can survive or by the rates at which toxic symptoms appear. This is seen, for example, in the work of Corner & Sparrow (1956) who compare the toxicity of various copper and mercury compounds to *Artemia* and some other Crustacea.

In general the results indicate that *Artemia* must possess a fair degree of permeability, and therefore that the ability to survive in concentrated brines must be due to the presence of regulatory mechanisms that actively adjust the composition of the body fluid.

SUMMARY

1. The survival of *Artemia salina* adults in various media has been studied.

2. Prolonged survival is only possible in media in which certain sodium salts (principally NaCl) predominate.

3. Certain substances are found to be highly toxic.

4. It is confirmed that the high toxicity of potassium ions can be antagonized by sodium ions.

5. The results indicate that the animal is appreciably permeable.

I wish to thank Dr J. A. Ramsay, F.R.S., for his interest and advice, and the Department of Scientific and Industrial Research for a maintenance grant.

REFERENCES

Abonyi, A. (1915). Experimentelle Daten zum Erkennen der *Artemia*-Gattung. *Z. wiss. Zool.* **114**, 95.

Baas Becking, L. G. M., Karstens, W. K. H. & Kanner, M. (1936). Salzeffekte und Milieu bei *Artemia salina* L. nebst Bermerkungen über Ionenantagonismus. *Protoplasma*, **25**, 32.

Barigozzi, C. (1946). Ueber die geographische Verbreitung der Mutanten von *Artemia salina* Leach. *Arch. Klaus-Stift. VererbForsch.* **21**, 479.

BOONE, E. & BAAS BECKING, L. G. M. (1931). Salt effects on eggs and nauplii of *Artemia salina* L. *J. gen. Physiol.* **14**, 753.

CORNER, E. D. S. & SPARROW, B. W. (1956). Observations on the poisoning of certain crustaceans by copper and mercury. *J. Mar. Biol. Ass.* **35**, 531.

DADAY DE DÉES, E. (1910). Monographie Systematique des Phyllopodes Anostracés. *Ann. Sci. Nat.* 9. *Zool.* **11**, 111.

GOLDSCHMIDT, E. (1952). Fluctuation in chromosome number in *Artemia salina*. *J. Morph.* **91**, 111.

JACOBI, E. F. & BAAS BECKING, L. G. M. (1933). Salt antagonism and effect of concentration in nauplii of *Artemia salina* L. *Tijdschr. ned. dierk. Ver.* (Ser. 3). **3**, 145.

KUENEN, D. J. (1939). Systematical and physiological notes on the brine shrimp, *Artemia*. *Arch. néer. Zool.* **3**, 365.

MARTIN, E. G. & WILBUR, B. C. (1921). Salt antagonism in *Artemia*. *Amer. J. Physiol.* **55**, 290.

STELLA, E. (1933). Phenotypical characteristics and geographical distribution of several biotypes of *Artemia salina* L. *Z. indukt. Abstamm.- u. Vererb.Lehre.* **65**, 412.

VINOGRADOV, A. P. (1953). *The Elementary Chemical Composition of Marine Organisms*. New Haven: Mem. Sears Found. Mar. Res. 2.

THE OSMOTIC AND IONIC REGULATION OF *ARTEMIA SALINA* (L.)

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INTRODUCTION

The branchiopod Crustacea are mainly confined to fresh or only slightly brackish waters. A few species have evolved a tolerance to more saline media (Beadle, 1943*a*, *b*). Of these *Artemia salina* is the most successful, and has a wide-spread distribution in salt pools and salt lakes of high salinities.

In a previous paper (Croghan, 1958*a*), the survival of *Artemia* in various media is described. Prolonged survival only occurs in media in which certain sodium salts (principally NaCl) predominate. In sea-water media *Artemia* can survive actively over an extremely wide concentration range.

Early work suggested that *Artemia* was hypotonic to concentrated media. Abonyi (1915) found that in animals from a brine of about 10% NaCl, the chloride, expressed as NaCl, was equivalent to only 0·8% of the original wet weight. Later Martin & Wilbur (1921) found a very low ash weight in *Artemia*, and concluded that the concentration of the body fluids must be well below that of the medium. Other work has been mainly on samples of haemolymph obtained from the extensive haemocoel. Medwedewa (1927) made a few determinations of the osmotic pressure, and concluded that the haemolymph was markedly hypotonic to the medium. Warren, Kuenen & Baas Becking (1938) measured the sodium and chloride concentrations in the haemolymph. Their paper is full of errors but they confirm that the haemolymph is hypotonic to the medium, and that NaCl is the most important osmotic constituent of the haemolymph. Kuenen (1939) continued this work, and extended somewhat the range of external concentration over which hypotonic regulation was known to occur. More recently Plattner (1955) measured the haemolymph osmotic pressure of *Artemia* from media containing from 2 to 28·5% total salts, and found a very marked hypotonicity even in the most concentrated media. He also found that in animals transferred to distilled water there was a fairly rapid fall in the haemolymph osmotic pressure. Another aspect was studied by Ussing (in Krogh, 1939), who found a rather slow exchange of D_2O. As the animal is therefore to some extent permeable, an osmo-regulatory mechanism must be present.

It is clear that *Artemia* has evolved a mechanism that maintains the haemolymph hypotonic in highly saline media. In the present paper the osmotic pressure and chemical composition of the haemolymph and the chemical composition of the whole animal in relation to the composition of the medium is described. Evidence concerning permeability and active transport will also be presented.

MATERIAL AND METHODS

Adult *Artemia* (about 10–12 mm. in length) from a sea-water culture described previously (Croghan, 1958*a*) were used. Experiments were done within the temperature range 18–24° C.

The animals have been acclimatized to a very wide range of external salinities. Low salinities were produced by diluting sea water, and higher salinities by boiling down sea water and re-aerating. Over a wide range the animals can be transferred from one medium to another with a low mortality incidence. In the more extreme ranges the mortality is greater, but is lessened if the animals are acclimatized over a period of days by gradually diluting or increasing the concentration of the medium. In the case of the most concentrated media (greater than 600% sea water) acclimatization was obtained by allowing evaporation to proceed until, after several weeks, NaCl began to crystallize.

Haemolymph samples

The animal was removed from its medium with a rubber teat pipette, rinsed rapidly in distilled water, dried on filter paper and cigarette paper, transferred to a slide and covered with liquid paraffin. A collecting pipette was made by drawing out a piece of 2 mm. diameter glass tubing to a sharp tip. The pipette was attached to a length of thin rubber tubing, the other end of which was held in the mouth. Liquid paraffin was sucked into the pipette. Working under a low-power binocular microscope, the body wall was punctured with a needle over the dorsal vessel, and the cherry-red haemolymph was sucked into the pipette. From a large adult 10–12 mm. long, and weighing about 8 mg., about 1–2 μl. of haemolymph could easily be obtained. The animals did not survive the sampling procedure. The sample was stored under liquid paraffin in a lacquered watch-glass. When it was not possible to analyse the samples immediately they were stored in a refrigerator or deep-freeze. Fortunately, the blood does not coagulate.

Whole animal samples

For each sample thirty to thirty-five animals were pipetted from their medium, rinsed quickly in distilled water, and dried on filter paper. Final traces of adherent water were removed by sprinkling the animals on the filter paper with a few drops of acetone, and finally drying them quickly with an air jet. The animals were transferred to a silica crucible and the wet weight was determined. The water content was found by difference after heating at 105° C. for 6–8 hr. The samples were then ashed at 550° C. for 30 min. For chloride determinations small quantities of either $KHCO_3$ or $NaHCO_3$ were added before ashing to reduce possible loss of this ion. 1 ml. of 20% sulphuric acid was added to the ash, and the extract was used for chemical analyses. The methods used were the same as for haemolymph except that larger micropipettes were used. The concentrations of sulphate and phosphate in the final solutions were well below the interference levels of the flame-photometer.

Osmotic pressure

This was determined using the micro-cryoscopic method of Ramsay & Brown (1955). Results were repeatable to within 0·01° C. Samples were usually done in duplicate or triplicate. The osmotic pressure was expressed empirically in terms of that concentration of NaCl solution that would give the same depression. Over the range 0–5 %NaCl, the freezing-point depression, Δ, was a linear function of concentration with a conversion factor of % NaCl $= \Delta/0{\cdot}60$.

Checks showed that there was no appreciable change in freezing-point of haemolymph stored under liquid paraffin and left at room temperature overnight. But whenever it was necessary to store a sample this was done at low temperature as an added precaution.

Chloride concentration

The first method of Ramsay, Brown & Croghan (1955) was used. For haemolymph samples micropipettes of 0·5–1 μl. proved most convenient. The pipettes were calibrated using standard NaCl solutions. With both standards and biological fluids the standard deviation of a series of titrations was not greater than 1 %. As far as possible titrations were done in duplicate or triplicate.

Sodium concentration

This was determined using an EEL flame-photometer. With haemolymph it was possible to do a determination on about 0·5–1 μl. A sample was transferred with a micropipette into 2 ml. of distilled water in a small polythene tube, and mixed by a gentle stream of air-bubbles. Standards were prepared in a similar way. The standard deviation of a series of similar samples giving nearly full-scale deflexion at maximum sensitivity was about 1–3 %. Whenever possible samples were done in duplicate or triplicate.

Potassium concentration

This also was determined with the EEL flame-photometer. The technique was the same as for sodium determinations. Rather large volumes of haemolymph had to be collected for a single determination (10–15 μl.), and this meant pooling the haemolymph from several individuals. It was not always possible to run a duplicate unknown, and thus the accuracy is likely to be somewhat less than in the case of sodium.

Magnesium concentration

This was determined by a modification of the method of Orange & Rhein (1951). A haemolymph sample (20–40 μl.) was deproteinized by adding an equal volume of 10% trichloracetic acid and centrifuging. The supernatant was pipetted off, and added to 1 ml. of a very dilute Titan yellow solution in a polythene tube. 1 ml. N-NaOH solution was then added. The tube was stoppered and well shaken, and the extinction relative to a blank was read off on a Spekker absorptiometer, using 2 cm. light path microcells, and green filters. A series of standards was also prepared. With the limited volumes of haemolymph available the method was being pushed to the limit of its sensitivity. High accuracy could not therefore be expected.

Phosphate content

Phosphate was not detectable in the available volumes of haemolymph, but estimations were made on the phosphate in the ash of whole animals. A modification of Delory's method (1949) was used. A mixture of 1 ml. of the aminonaphthol-sulphonic acid reagent and 2 ml. of the acid ammonium molybdate reagent was diluted to 20 ml. 1 ml. of this mixture was then placed in each of a series of tubes, and a measured volume of unknown or of one of a series of standards was added to each with a micropipette. The extinction was determined on a Spekker using 2 cm. light path microcells and red filters. The standard deviation of a series of similar standards was less than 1%.

RESULTS

Composition of the haemolymph

Animals were left in a new sea-water medium for at least 2 days, and in many cases considerably longer before the haemolymph was sampled. The samples were either from single animals, or were pooled samples from up to twenty animals or more.

The haemolymph osmotic pressure was determined. A sample of the medium was also measured in comparison. The more concentrated media had to be diluted considerably to bring them within the range of the thermometer. The results were then multiplied by the dilution factor. The highest values for the medium are apparently above the maximum solubility of NaCl. This presumably means that a crystallizing sea-water brine has a higher osmotic pressure than a saturated NaCl solution. Thus, an apparent anomaly appears when the osmotic pressure is expressed in terms of NaCl.

The results are plotted out on two graphs (Figs. 1, 2) to cover the very wide external salinity range. Also included in these graphs for convenience are data on the osmotic pressure of the gut fluids. This will be discussed in a subsequent paper (Croghan, 1958*c*).

The results extend the range and precision of the observations by earlier workers, and demonstrate clearly the relative constancy of the haemolymph concentration and its independence from that of the medium. Over a range of medium concentration increasing by a factor of about 100, the haemolymph concentration increases by a factor of only about 6. Although this increase is relatively small, it still indicates that a considerable toleration by the tissues to changes in total haemolymph concentration is required.

In the more concentrated media the hypotonicity of the haemolymph is very marked. In media more dilute than 25% sea water, *Artemia* is hypertonic to its medium. It is behaving like a brackish-water organism. But the most dilute sea-water medium to which it has been possible to adapt *Artemia*, even over a period of several weeks, is still only as dilute as 0·26% NaCl. In fresh water, *Artemia* dies in about 24 hr.

The osmotic pressure of the haemolymph of *Artemia* from dilute media is not much different from that of the fresh-water species *Chirocephalus diaphanus*. In this animal Panikkar (1941*a*) found a range of haemolymph osmotic pressure of 0·44–0·50% NaCl. In the present work haemolymph samples from three large specimens were tested separately. The mean osmotic pressure was found to be 0·43% NaCl.

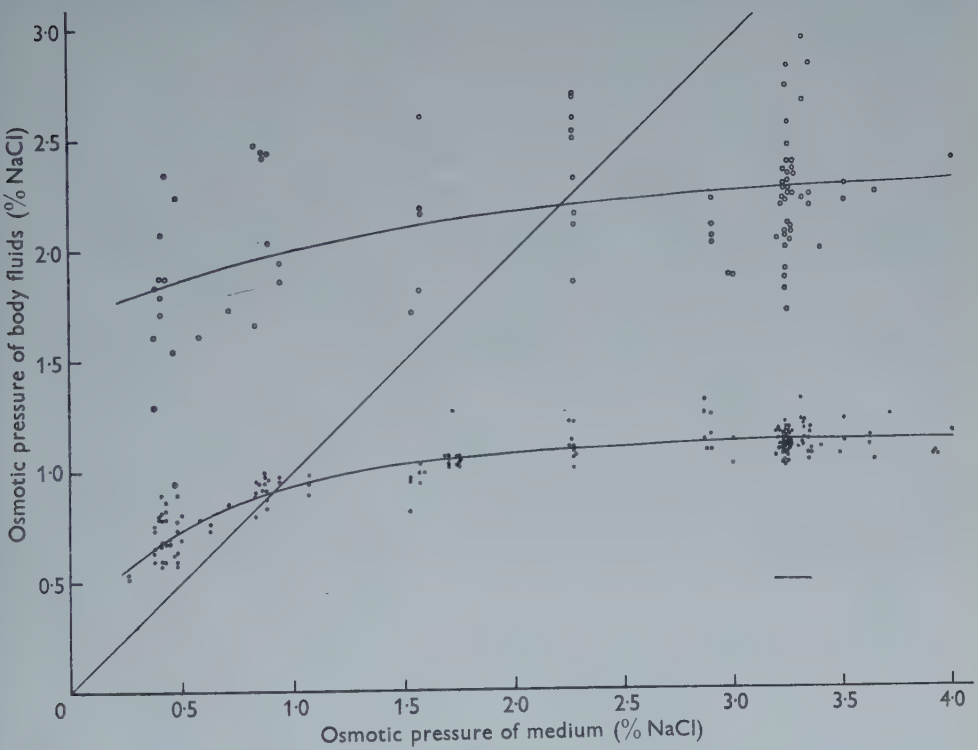

Fig. 1. The relation between the osmotic pressure of the body fluids and the medium in the more dilute media. Haemolymph osmotic pressure, ●; gut fluid osmotic pressure, ○. The short horizontal line represents the range of values of ordinary sea water. The diagonal line through the origin represents isotonicity.

In view of the marked hypotonicity of *Artemia* adults in concentrated media, it was considered of interest to examine nauplii. Haemolymph has been obtained from a few 2nd instar nauplii (criteria of Heath, 1924). A nauplius (0·5–0·8 mm. long) was pipetted from the sea-water medium, in which it had hatched, onto a slide, the adherent sea water was carefully removed with cigarette paper fragments, and the nauplius was covered by a drop of liquid paraffin. The neck organ was punctured with the fine silica capillary. A haemolymph sample was drawn up, and the osmotic pressure determined. The sea-water medium was found to be 3·32% NaCl, and the mean result for the haemolymph concentration of five nauplii was $1{\cdot}30 \pm 0{\cdot}17$% NaCl. These results clearly indicate that the nauplii are hypotonic to sea water, and have a haemolymph concentration fairly similar to that of the adult.

Chemical analyses have been carried out on many of the haemolymph samples whose osmotic pressures are recorded in Figs. 1 and 2. In many cases several ions were determined in the same pooled sample, and this was particularly so in the case of sodium and chloride. Information has been obtained for animals acclimatized to a range of external salinities varying from 10 to 600% sea water. For convenience and clarity these results are presented as a function of haemolymph osmotic pressure (Fig. 3).

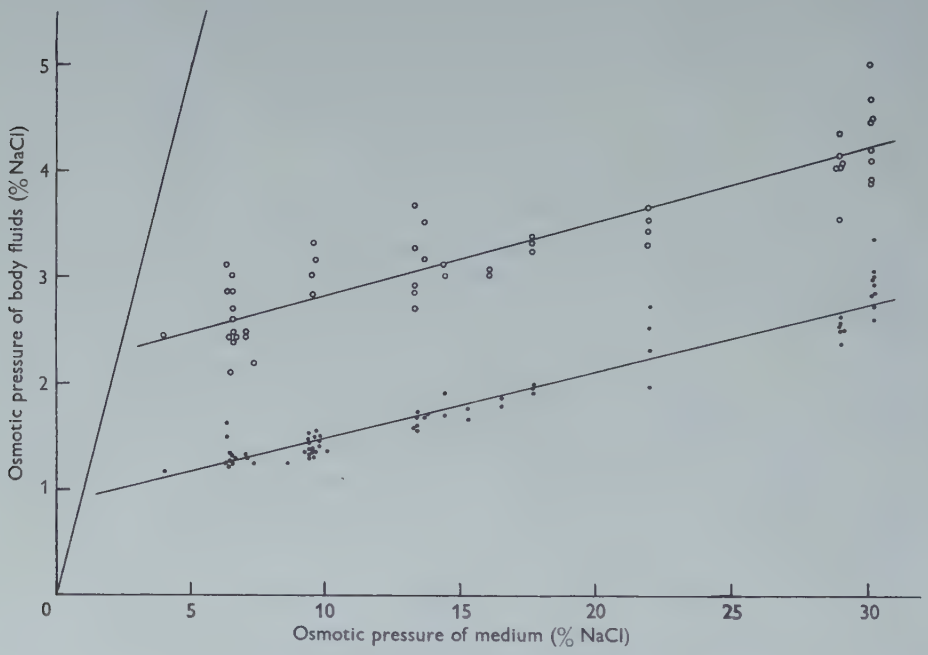

Fig. 2. The relation between the osmotic pressure of the body fluids and the medium in the more concentrated media. Haemolymph osmotic pressure, ●; gut fluid osmotic pressure, ○. The diagonal line through the origin represents isotonicity.

It is clear that ionized sodium salts can account for about 90% of the total haemolymph osmotic pressure. The chloride equivalence is some 10% lower. These two ions account for almost all the observed osmotic pressure (as is probably general in Crustacea), and the ratio of their concentrations does not change appreciably over the range of haemolymph osmotic pressure studied. Potassium and magnesium ions are very much less important osmotic constituents.

Chemical analyses were also carried out on some of the media from which the animals were taken. As these media covered a wide range of osmotic pressure, the data have been adjusted to the osmotic pressure of ordinary sea water. The mean results are as follows: osmotic pressure of sea water, $\Delta = 1{\cdot}95°$ C. (= 3·25% NaCl); chloride, 565 mM./l.; sodium, 485 mM./l.; potassium, 11 mM./l.; magnesium, 55 mM./l.

The haemolymph ionic ratios are very different from those of the medium. The Na:Cl ratio of the sea-water brine is 0·86, whereas in the haemolymph the ratio is always greater than one. The haemolymph magnesium concentration is extremely low, and is well below that of the sea-water brines (Fig. 4). The potassium concentration is held relatively constant in the haemolymph, and can be very different

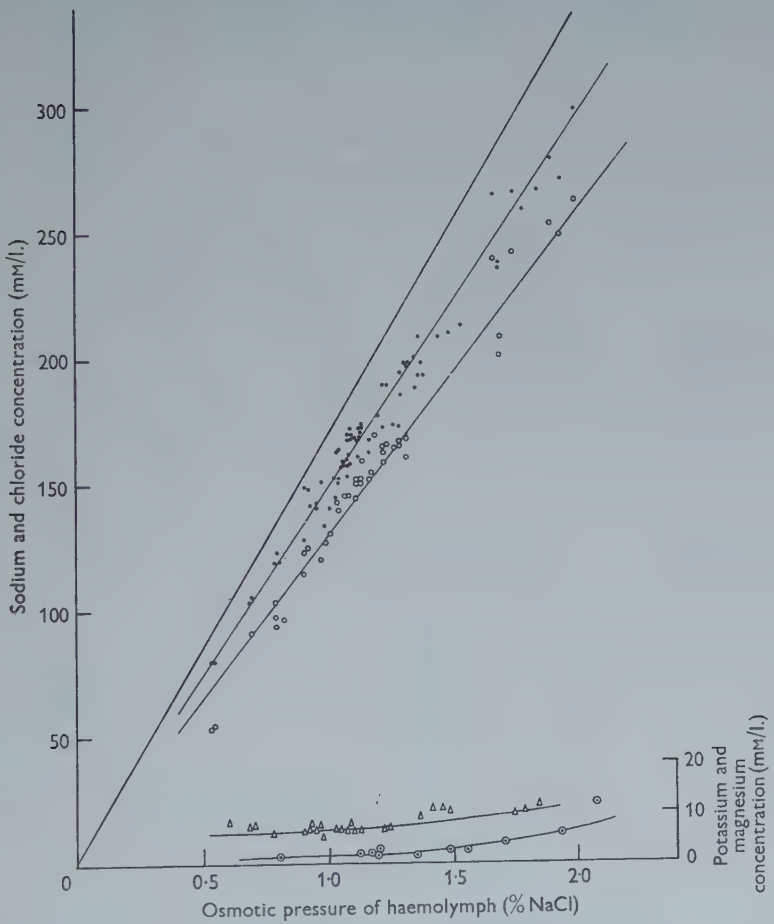

Fig. 3. The chemical composition of the haemolymph. Sodium, ●; chloride, ○; potassium, △; magnesium, ⊙. The diagonal line through the origin represents the values expected if all the haemolymph osmotic pressure was accounted for by sodium and chloride ions.

from that of the medium (Figs. 3, 4). The ratio K:Na in the haemolymph compared with that in the medium ($K_i \times Na_m / Na_i \times K_m$) is also plotted in Fig. 4. This enrichment factor is always greater than unity, even when the actual potassium concentration in the medium is well above that in the haemolymph. This relative elevation of the haemolymph K:Na ratio is a further example of one of the most widespread features of animal blood.

Shortage of material prevented a detailed comparative study of the haemolymph of fresh-water branchiopods. However, a few measurements were made on *Chirocephalus diaphanus* from fresh water. Haemolymph samples from the three large specimens previously mentioned were analysed separately. The mean values are: osmotic pressure, 0·43 % NaCl (73·5 mM./l.); sodium, 62 mM./l.; chloride, 51 mM./l. The Na : Cl ratio, and the contribution of these two ions to the total osmotic pressure in *Artemia* are very similar to these *Chirocephalus* values.

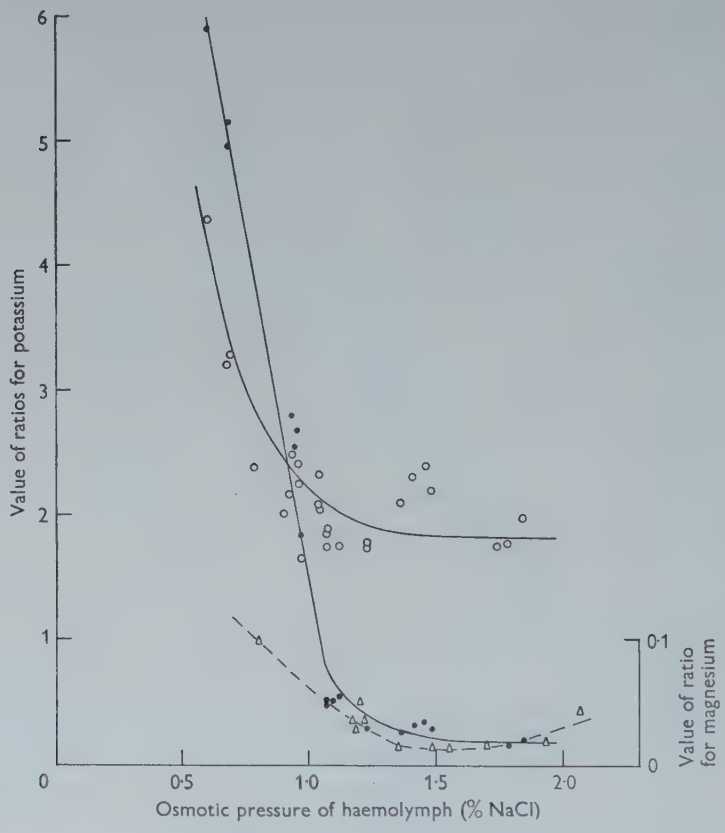

Fig. 4. The values of some ionic ratios. K_i/K_m, ●; $K_i \times Na_m/Na_i \times K_m$, ○; Mg_i/Mg_m, △. (i is haemolymph and m is medium.)

The curves relating the osmotic pressure of *Artemia* haemolymph to that of the medium (Figs. 1, 2) have a definite slope, and these changes in haemolymph osmotic pressure are due to changes in the NaCl concentration in the haemolymph (Fig. 3). This is evidence of permeability. But, as the haemolymph composition is still very different from the medium, the haemolymph steady state must be maintained by active mechanisms that balance the passive diffusion movements of water and ions. This becomes clearer if we analyse more closely some of the data included in Figs. 1–3. In the experiment recorded in Table 1, the osmotic pressure and sodium

concentration were both determined on the haemolymph from single large animals. Group 1 were animals from a culture that had been kept in 50% sea water for 4 days. The rest of the animals were then transferred to 300% sea water. After 2 days haemolymph samples were taken (Group 2). The remaining animals were then divided into two groups. Group 3 was left in the 300% sea water for a further 2 days before sampling, and group 4 was transferred back to a 50% sea water, and left for 3 days before sampling. During the experiment the mortality due to transferring the animals to media of different concentration was extremely low, and thus selection

Table 1. *The osmotic pressure and sodium concentration of the haemolymph of individual animals*

Group	Medium		Haemolymph	
	Osmotic pressure (% NaCl)	Na mM./l.	Osmotic pressure (% NaCl)	Na mM./l.
1	1·75	246	1·05 ±0·02 (6)	156± 6 (6)
2	9·37	1380	1·39 ±0·06 (7)	205± 8 (7)
3	9·37	1380	1·36 ±0·06 (4)	197±10 (4)
4	1·70	242	1·055±0·02 (5)	156± 3 (5)

The mean values and the standard deviations of each group of individual animals are given. The figures in brackets are the number of individuals studied.

Table 2. *The effect of distilled water on the composition of the haemolymph*

Medium	Haemolymph			
	Sample group	Osmotic pressure (% NaCl)	Na mM./l.	Cl mM./l.
Original sea-water medium (osmotic pressure 3·63% NaCl)	1	1·125	172	153·5
	2	1·16	169	153·5
Distilled water (frequently changed) 13 hr. exposure	3	0·59	79·5	71
	4	0·615	72	60

could not have influenced the results. On transferring animals to a higher concentration the osmotic pressure rises slightly but definitely, due to an increase in sodium salts, to a new steady state. This new level is reached and stabilized within 2 days. On transferring some animals back to the dilute medium, the haemolymph osmotic pressure and sodium concentration fall to the original level again, although the osmotic pressure and sodium concentration of this medium is still above that of the haemolymph. This experiment clearly demonstrates permeability, and that the animal can actively decrease the haemolymph osmotic pressure whilst still in a hypertonic medium.

In glass-distilled water, *Artemia* dies in about 24 hr. In Table 2 some haemolymph analyses are given. Each group refers to a sample pooled from several individuals.

It seems clear that death in distilled water is due to a rapid loss of NaCl and/or gain of water. This demonstrates a considerable degree of permeability, and indicates that in dilute media, to which *Artemia* is hypertonic, active mechanisms for taking up NaCl and excreting excess water must be present.

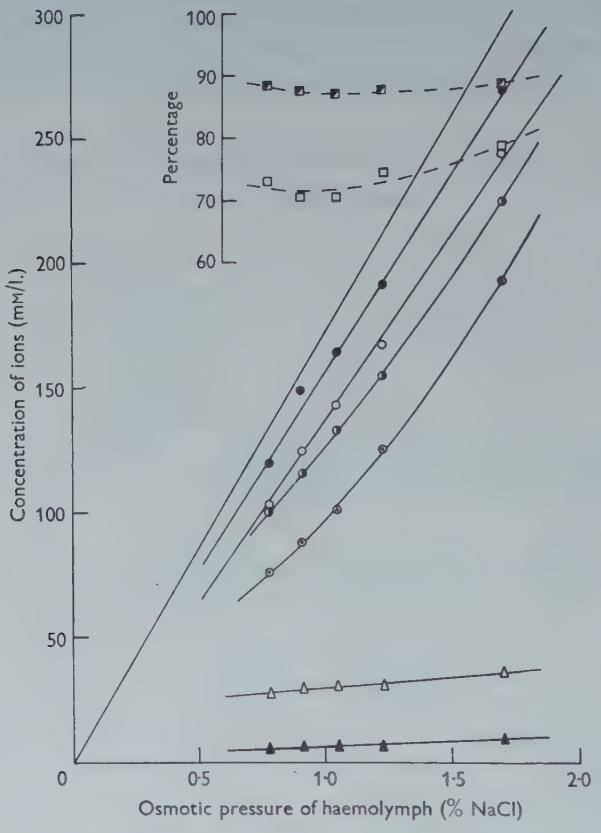

Fig. 5. The chemical composition of the whole animal in comparison with the haemolymph. Sodium concentration in haemolymph, ●; in T.B.W., ◑. Chloride concentration in haemolymph, ○; in T.B.W., ⊙. Potassium concentration in haemolymph, ▲; in T.B.W., △. T.B.W. as percentage of original weight, ◩. Chloride space as percentage of T.B.W., □. (T.B.W. is total body water.)

Composition of the whole animal

Animals were acclimatized for several days in clean filtered media varying from 15–500% sea water. Two series of samples were prepared. One ashed with $KHCO_3$ was used for chloride and sodium estimations, and the other ashed with $NaHCO_3$ was used for chloride and potassium estimations. Results are expressed as mM. per litre of total body water (T.B.W.). Comparative analyses were also made on the haemolymph of groups of animals removed from the media at the same time as the samples for ashing.

The results are plotted in Fig. 5. The percentage of water in the animal remains

fairly constant. The concentrations of sodium and chloride in the T.B.W. rise closely parallel to and not much below the haemolymph values. If the chloride is assumed to be entirely extracellular, the chloride space (Cl conc. in T.B.W./Cl conc. in haemolymph) gives the haemolymph volume (extracellular space). This chloride space is plotted in Fig. 5. It is relatively constant and very high. The ease with which large amounts of haemolymph can be obtained also indicates a large haemolymph space. The animal gives the impression of being an elastic sac, in which shape and mechanical rigidity are maintained by haemolymph pressure, the actual tissues occupying a relatively small volume. This is confirmed by the fact that, although the potassium concentration in the T.B.W. is higher than in the haemolymph, as one would expect for a mainly intracellular ion, its concentration in the T.B.W. is still quite low.

It is of interest to determine how the changes in external salinity affect the total water and ion content of the animals, as distinct from concentration. For this purpose the phosphate content of the acid ash extracts, used to obtain the data for Fig. 5, was also determined. As there is very little phosphate in the haemolymph or the medium and large amounts in the ash extracts, it is clear that the phosphate is derived from intracellular phosphorus compounds. It is reasonable, therefore, to consider that the phosphate content of the ash is unlikely to have been affected by the various external salinities used in these experiments. Changes in the ratios of total water content and the total amount of various ions present to phosphate content therefore indicate net movements of these substances into or out of the animal. The mean values for the animals from the most dilute medium used (osmotic pressure = 0·48% NaCl) have been put equal to 100, and the ratios for the animals from the other more concentrated media have been expressed as percentages of this. The results are presented in Fig. 6. It is clear that the changes in haemolymph osmotic pressure in different media are accounted for more by changes in the NaCl content of the animal than by changes in water content, and this is direct evidence that the animal can actively excrete NaCl against the concentration gradient. The potassium of the animal, as would be expected for a mainly intracellular ion, only rises slightly in the increased external salinities.

Localization of permeability

The preceding sections have indicated that *Artemia* is permeable. Further information was obtained by placing animals in a mixture of sea water and glycol. Animals from a sea-water culture (= 3·24% NaCl) were placed in sea water plus 15% (v/v) glycol and a little phenol red. In this medium they survived well for the duration of the experiment. Some of the animals were previously ligatured tightly at the neck and anus with fine fibres teased out of strands of bolting silk. Such ligatured animals have survived with active swimming movements for a day or more. The phenol red showed that the unligatured animals were actively swallowing the medium, whereas the ligatured ones could not do so. Haemolymph samples were easily obtained from both unligatured and ligatured animals, and there was no sign of appreciable dehydration in either.

The results are graphed in Fig. 7. Each point represents a single animal. The osmotic pressure rises far more rapidly in the unligatured animals than in the ligatured animals. The results demonstrate a considerable degree of permeability. The ligaturing experiment clearly demonstrates that most of this permeability is

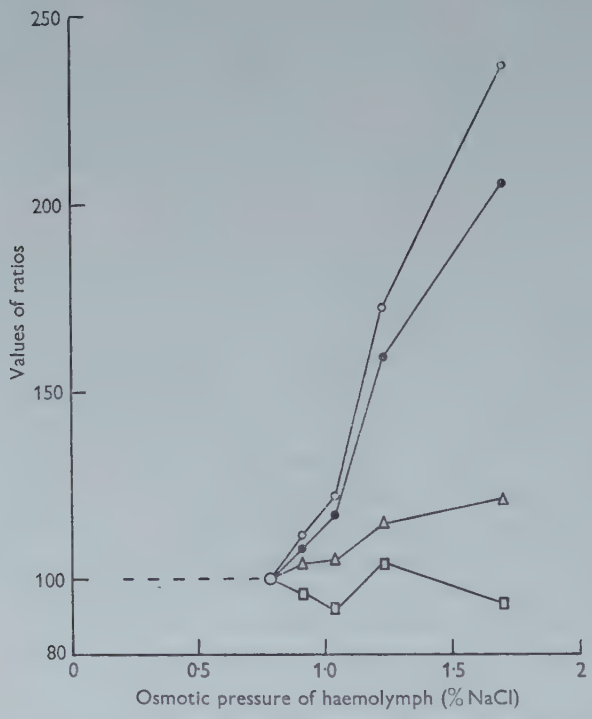

Fig. 6. The chemical composition of the whole animal relative to phosphate content. Sodium/phosphate, ●; chloride/phosphate, ○; potassium/phosphate, △; water/phosphate, □.

across the gut epithelium, the external surface being much less permeable. After 23 hr. exposure, the haemolymph chloride concentration was also determined in five ligatured animals, and in the five unligatured animals that had the highest haemolymph osmotic pressure. The results are given in Table 3. It is clear that the rise

Table 3. *The composition of the haemolymph of some animals after 23 hr. in the medium containing sea water and glycol*

Unligatured animals		Ligatured animals	
Osmotic pressure (% NaCl)	Cl mM./l.	Osmotic pressure (% NaCl)	Cl mM./l.
3·85	149	1·35	137
3·67	144	1·44	123
3·89	142	1·72	116
3·00	152	1·35	137
3·52	142	1·53	104

in osmotic pressure in the unligatured animals is not due to osmotic dehydration and a resultant rise in chloride concentration, but must be due to the entry of the relatively large glycol molecules across the gut epithelium.

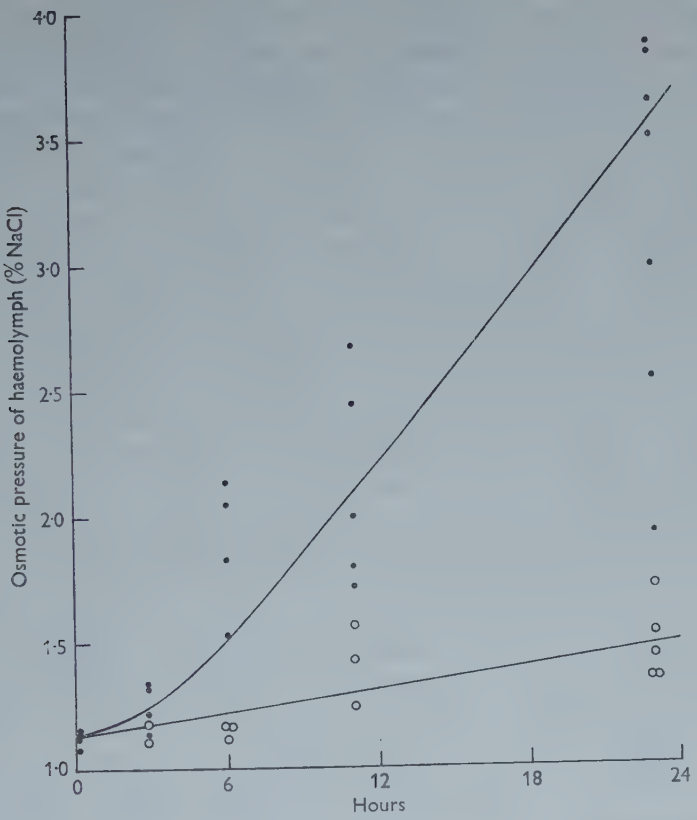

Fig. 7. The effect of a medium containing sea water and glycol on the osmotic pressure of the haemolymph. Normal unligatured animals, ●; ligatured animals, ○.

DISCUSSION

It has been shown that the osmotic pressure and ionic composition of *Artemia* haemolymph can be very different from the medium. Even in concentrated media, the haemolymph osmotic pressure is still less than that of a typical marine invertebrate in sea water, and is more like that of a fresh-water animal in fresh water. Further, the ionic composition of the haemolymph has characters more like those of a fresh-water animal, e.g. a high Na:Cl ratio, and an extremely low magnesium concentration. All this is physiological confirmation of a fresh-water ancestry, and indicates that the evolution of *Artemia* has been a process of superimposing upon a basically fresh-water type of physiology a method of stabilizing the haemolymph composition at a level which the cells and tissues can tolerate. This has enabled the animal to colonize extremely saline media that are ecologically open habitats.

Artemia, contrary to the statement of Beadle (1943*a*), also possesses the ability to maintain a hypertonic haemolymph in dilute media, but this ability is limited and appears more a 'vestigal character', and the animals cannot survive long in fresh water or distilled water.

A number of other animals have been shown to be markedly hypotonic to saline media. These include *Aedes detritus* larvae (Beadle, 1939), the marine teleosts (Smith, 1932), and certain palaemonids (Panikkar, 1941*b*). The ability of these animals to live in concentrated media is appreciably less well developed than in *Artemia*, although *Aedes detritus* can live in brines up to at least 10% NaCl (Beadle, 1939). All these hypotonic forms seem to be of fresh-water origin, and to have had a similar evolutionary history to *Artemia*.

The experiments have shown that *Artemia* is appreciably permeable. In hypertonic media *Artemia* would tend constantly both to gain NaCl and to lose water, and, it is clear that in these media the haemolymph steady state must be maintained by well-developed active transport mechanisms both for excreting NaCl and for taking up water. The demonstration that the animal can decrease the osmotic pressure and NaCl concentration of the haemolymph and the NaCl content of the whole animal whilst still in a hypertonic medium is direct evidence for such mechanisms.

The low permeability of the external surface of *Artemia* may be regarded as an adaptation hindering passive diffusion of NaCl and water. Most of the permeability of the animal is across the gut epithelium, and it seems clear that in hypertonic media it is across this epithelium that there will be the greatest tendency for NaCl to enter and for water to leave the haemolymph. A considerable permeability across the gut epithelium would be expected as various products of digestion have to be taken up and as the cuticle which covers the external surface is in the gut only represented by a thin peritrophic membrane. In *Aedes detritus* larvae Beadle (1939) found that the permeability was similarly restricted.

The nature of the active transport mechanisms in *Artemia* in comparison with those in other organisms showing hypotonic regulation is of considerable interest and will be discussed in subsequent papers (Croghan, 1958*b*, *c*).

SUMMARY

1. It has been possible to adapt *Artemia* to sea-water media varying from 0·26% NaCl to crystallizing brine. In fresh water or distilled water survival is relatively short.

2. The osmotic pressure of the haemolymph is relatively independent of the medium and increases only slightly as the medium is made more concentrated. In the more concentrated media the haemolymph is very markedly hypotonic. In media more dilute than 25% sea water the haemolymph is hypertonic. In distilled water there is a rapid fall of haemolymph concentration. The haemolymph of nauplii from sea water is hypotonic.

3. The sodium, potassium, magnesium, and chloride concentrations of the haemolymph have been determined. The bulk of the haemolymph osmotic pressure

is accounted for by sodium and chloride ions. The ionic ratios of the haemolymph are relatively constant, and very different from those of the medium.

4. The concentrations of ions in the whole animal have been studied. The chloride space is extremely high. Such changes in haemolymph osmotic pressure that do occur as the medium concentration is varied are due more to net movements of NaCl into or out of the body than to water movements.

5. Evidence is collected to show that an appreciable degree of permeability exists. Most of this permeability is localized to the gut epithelium, the external surface being much less permeable.

6. It is clear that *Artemia* must possess mechanisms that can actively excrete NaCl and take up water in hypertonic media. It has been demonstrated that *Artemia* can lower the haemolymph osmotic pressure by excreting NaCl from the haemolymph against the concentration gradient.

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REFERENCES

Abonyi, A. (1915). Experimentelle Daten zum Erkennen der *Artemia*-Gattung. *Zeits. wiss. Zool.* **114**, 95.

Beadle, L. C. (1939). Regulation of the haemolymph in the saline water mosquito larva *Aedes detritus* Edw. *J. Exp. Biol.* **16**, 346.

Beadle, L. C. (1943*a*). Osmotic regulation and the fauna of inland waters. *Biol. Rev.* **18**, 172.

Beadle, L. C. (1943*b*). An ecological survey of some inland saline waters of Algeria. *J. Linn. Soc. Zool.* **41**, 218.

Croghan, P. C. (1958*a*). The survival of *Artemia salina* (L.) in various media. *J. Exp. Biol.* **35**, 213.

Croghan, P. C. (1958*b*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. *J. Exp. Biol.* **35**, 234.

Croghan, P. C. (1958*c*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut. *J. Exp. Biol.* **35**, 243.

Delory, G. E. (1949). *Photoelectric Methods in Clinical Biochemistry*. Hilger and Watts. London.

Heath, H. (1924). The external development of certain phyllopods. *J. Morph.* **38**, 453.

Krogh, A. (1939). *Osmotic Regulation in Aquatic Animals*. Cambridge.

Kuenen, D. J. (1939). Systematical and physiological notes on the brine shrimp, *Artemia*. *Arch. néer. Zool.* **3**, 365.

Martin, E. G. & Wilbur, B. C. (1921). Salt antagonism in *Artemia*. *Amer. J. Physiol.* **55**, 290.

Medwedewa, N. B. (1927). Über den osmotischen Druck der Hämolymphe von *Artemia salina*. *Z. vergl. Physiol.* **5**, 547.

Orange, M. & Rhein, H. C. (1951). Microestimation of magnesium in biological fluids. *J. Biol. Chem.* **189**, 379.

Panikkar, N. K. (1941*a*). Osmotic behaviour of the fairy shrimp *Chirocephalus diaphanus* Prévost. *J. Exp. Biol.* **18**, 110.

Panikkar, N. K. (1941*b*). Osmoregulation in some palaemonid prawns. *J. Mar. Biol. Assoc.* **25**, 317.

Plattner, F. (1955). Der osmotische Druck von *Artemia salina*. *Pflüg. Arch. ges. Physiol.* **261**, 172.

Ramsay, J. A. & Brown, R. H. J. (1955). Simplified apparatus and procedure for freezing-point determinations upon small volumes of fluid. *J. Sci. Instrum.* **32**, 372.

Ramsay, J. A., Brown, R. H. J. & Croghan, P. C. (1955). Electrometric titration of chloride in small volumes. *J. Exp. Biol.* **32**, 822.

Smith, H. W. (1932). Water regulation and its evolution in fishes. *Quart. Rev. Biol.* **7**, 1.

Warren, H., Kuenen, D. & Baas Becking, L. G. M. (1938). On the relation between internal and external medium in *Artemia salina* (L.) var. principalis Simon. *Proc. K. Akad. Wet. Amst.* **41**, 873.

THE MECHANISM OF OSMOTIC REGULATION IN *ARTEMIA SALINA* (L.): THE PHYSIOLOGY OF THE BRANCHIAE

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INTRODUCTION

In a previous paper (Croghan, 1958*b*) the very great osmo-regulating ability of *Artemia* has been described. It is clear that in concentrated media the animal must have very well-developed active mechanisms both for excreting NaCl and for taking up water to compensate for the passive movements of these substances into and out of the animal.

The staining of localized parts of Crustacea following immersion in dilute $AgNO_3$ solutions and subsequent reduction has been interpreted by Koch (1934) and Krogh (1939) as indicating the site of active ion uptake. But similar staining occurs in *Artemia* (Dejdar, 1930), and yet this animal in its normal hypertonic medium must be actively excreting NaCl (Croghan, 1958*b*). In this paper further observations on the branchiae of *Artemia* in relation to the mechanism of osmotic regulation are described.

MATERIAL AND METHODS

Adult *Artemia* as described previously (Croghan, 1958*a*) were used.

The basic silver-staining technique is very simple. Animals from a sea-water culture were used for most experiments. The living animal was washed in several changes of distilled water (for 1–2 hr. usually) to remove adherent chloride, and was then placed in 10^{-2} M-$AgNO_3$ solution for 2–5 min. The animal was then given a prolonged wash in several changes of distilled water (1–2 hr. usually) to remove any adherent $AgNO_3$, and was placed in photographic developer (1. I.D. 13.) for 1–2 min. to reduce to the black metallic state any silver that had been taken up.

The methods used in studying the composition of the haemolymph and of whole animal have been described previously (Croghan, 1958*b*).

RESULTS

Histochemistry of the branchiae

The only parts of *Artemia* that showed silver staining were the branchiae (metepipodites) of the first ten pairs of phyllopods. The last (eleventh) pair of branchiae and the rest of the animal never stained. The first ten pairs of branchiae became white in the $AgNO_3$ solution. In the developer these branchiae became intensely black. Microscopic observation showed that the blackening was granular.

Staining occurred whether the medium from which the animal was originally taken was hyper-, iso-, or hypotonic to the haemolymph, although the intensity did tend to be somewhat greater when the external salinity had been higher.

Most observations were made using 10^{-2} M-$AgNO_3$, but the most dilute solution used, 10^{-4} M-$AgNO_3$, still gave a similar result, although the duration of exposure to get a comparable effect was much greater (> 20 min.).

The fact that the branchiae turned white in the $AgNO_3$ solution indicated that the silver ions had entered and been precipitated. Some of these white-gill animals were washed in distilled water and then soaked in 5% HNO_3 (30 min.), washed well with water and then developed. In another experiment some living animals were washed and placed directly in 10^{-2} M-$AgNO_3$ made up in 5% HNO_3, and then washed thoroughly and developed. In both cases the branchiae were still blackened in the developer, suggesting strongly that the initial white precipitate is AgCl. The source of the chloride ion is of some interest. The period of washing in distilled water before transferring the animals to the $AgNO_3$ solution was varied from 3 min to 18 hr. Very prolonged washing resulted in some decrease of subsequent blackening, but the blackening was still considerable, and this suggested that the chloride was not derived directly from the medium, but was coming from the animal itself.

Close microscopical examination of isolated branchiae suggested that the black granular staining is very superficial. This was confirmed by watching branchiae becoming decolourized in Farmer's solution (potassium ferricyanide + hypo). The staining was still very superficial even when the violent treatment of development and subsequent Bouin fixation had caused the epithelium to retract away from the cuticle. This suggested that the black silver deposit was within the cuticle, which is only about 1 μ thick. In some cases the epithelium had distorted and retracted so much that the thin blackened cuticle could be pulled off the branchia like a loose glove; the epithelium thus seen was not stained. Further, if detached stained branchiae were heated with saturated KOH solution in a boiling water-bath for 3–4 hr the appearance of the silver stain was not affected, although on subsequent removal of the silver with Farmer's solution, it was seen that the branchial epithelium had entirely disappeared leaving only the thin transparent cuticular ghost. These experiments indicate that the silver staining is confined to the thickness of the cuticle, and does not appear to affect the underlying epithelium.

The effect of metabolic inhibitors was studied. Animals were immersed in 15% ethyl urethane in distilled water for periods of up to 6 hr. They were then placed for 5 min. in 10^{-2} M-$AgNO_3$ made up in 15% ethyl urethane solution, washed thoroughly and developed. Other animals were immersed in sea water containing 2% sodium azide for 3–18 hr., and then washed for times varying from 1–30 min to remove azide (which would precipitate silver ions). They were then placed in 10^{-2} M-$AgNO_3$ for 5 min, washed and developed. With both urethane and azide the animals were completely and irreversibly motionless and apparently dead long before they were transferred to the $AgNO_3$ solutions. Yet in both cases the first ten pairs of branchiae were still very black. This suggests that the silver staining is a purely passive process.

All these results with the silver technique indicate that the cuticle over the first ten pairs of branchiae is permeable, as silver ions and subsequently developer could not otherwise enter. The silver ions diffuse into the permeable cuticle, meet chloride ions derived from the animal via the branchial epithelium, and form an AgCl precipitate within the cuticle. The whole phenomenon is purely passive. Over the last pair of branchiae and the rest of the external surface of the animal the cuticle must be sufficiently impermeable to prevent this from occurring.

The effects of a few other substances on the branchiae of *Artemia* have been studied. Some *Artemia* were put into a saturated solution of methylene blue for about 4 hr. After removal and rinsing, the first ten pairs of branchiae were seen to be markedly blue. Apart from a little dye that might have entered the gut lumen, no other part of the animal was appreciably coloured. Detached phyllopods were examined microscopically. At the edge of the flattened branchiae it could be seen that the dye had diffused across the very thin cuticle, and had caused a granular blue staining in the epithelial cells that form a layer 8–16 μ thick under the cuticle. Other *Artemia* have been exposed to saturated $KMnO_4$ solution for 5–15 min. The first ten pairs of branchiae had turned brown. The rest of the animal appeared unaffected. Microscopical examination showed that the epithelium under the cuticle of these branchiae had become brown and distorted, and in many cases had pulled right away from the cuticular sac. The observations with both these substances also strongly support the view that the permeability of the external cuticle is localized to the first ten pairs of branchiae. It is only here that the methylene blue and $KMnO_4$ can enter and affect the underlying cells.

The results with $AgNO_3$, methylene blue and $KMnO_4$ are not direct evidence that active uptake or excretion is occurring, but they show where it could be occurring, as over the epithelium concerned with this process the cuticle must of course be permeable.

The fresh-water *Chirocephalus* also shows silver-staining of its branchiae (Dejdar, 1930; Panikkar, 1941*a*), and Panikkar claimed from this that the branchiae were the site of ion uptake. In the present work two large specimens (12–15 mm.) were studied. They were treated with 10^{-2} M-$AgNO_3$ and developed in the way described previously for *Artemia*. The first ten pairs of branchiae became stained as in *Artemia*. The last pair of branchiae, which were smaller than the preceding ones, were not stained.

Experimental destruction of the osmo-regulatory mechanism

Further information about branchial function was obtained by studying animals whose branchiae had been 'burnt' with $KMnO_4$ as just described. The animals were immersed for 5 min. in saturated $KMnO_4$ solution, and then removed and washed in 25% sea water. Many animals survived, and the only sign of damage was the browning and distortion of the epithelium of the first ten pairs of branchiae. These animals were kept in 25% sea water, which is approximately isotonic with the haemolymph of normal animals (Croghan, 1958*b*). Many died during the first 24 hr., but a considerable number survived well for a week or more, swimming, feeding, and reproducing in an apparently normal manner. In these survivors

the damaged branchial epithelium underwent a further slow degeneration and blackening.

The physiology of active survivors was studied. After at least 24 hr. in 25% sea water they were transferred to other media. The limits of survival were very much more restricted than for normal animals and ranged from only about 10–75% sea water. The haemolymph osmotic pressure was measured after about 48 hr. in these media. The results are plotted in Fig. 1, and should be compared with those with normal animals (Croghan, 1958*b*, figs. 1, 2). The 'burnt' animals had completely lost the ability to osmo-regulate, and were very closely isotonic with the medium. The upper limit of survival is set by the maximum haemolymph concentration that the tissues can tolerate. This concentration is approximately that of 75% sea water, and is about the same as the haemolymph concentration of a normal animal in a nearly saturated brine.

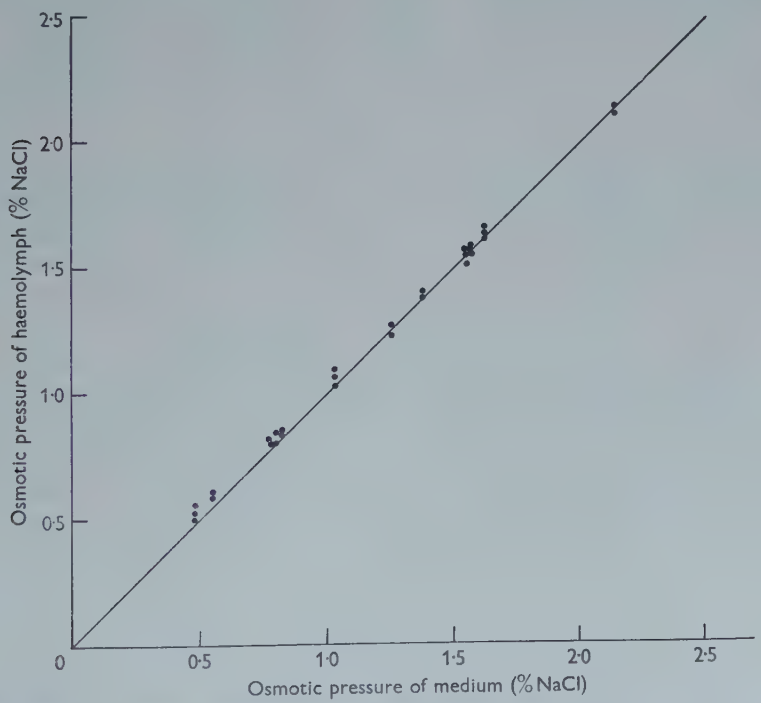

Fig. 1. The osmotic pressure of the haemolymph of animals treated with $KMnO_4$. The diagonal line represents the condition of complete isotonicity between the haemolymph and the medium.

There appears to be no recovery of osmo-regulatory function; some 'burnt' animals that had survived actively for a week in 25% sea water and had then been transferred to 40% sea water became closely isotonic with their new medium within 24 hr.

The chemical composition of the haemolymph of some of the 'burnt' animals whose osmotic pressure is recorded in Fig. 1 was studied. 'Burnt' animals after

24 hr. in 25% sea water were placed in 50% sea water for 48 hr. Haemolymph samples were obtained from active animals with black or brown branchiae. Three samples, each derived by pooling the haemolymph from two animals, were obtained. The results are summarized in Table 1. Although the haemolymph osmotic pressure had risen and become isotonic with the medium, the ionic ratios were still very similar to those of normal undamaged animals (Croghan, 1958*b*, fig. 3), and very different from those of the medium. In another experiment, 'burnt' animals

Table 1. *The haemolymph composition of animals 'burnt' with* $KMnO_4$

Material	Osmotic pressure (% NaCl)	Na mM./l.	Cl mM./l.
50% sea water	1·55	228	264
Haemolymph groups			
1	1·58	244	210
2	1·52	238	200
3	1·55	238	210

that had been in 25% sea water for 24 hr. were transferred to 40% sea water to which a little solid $MgSO_4$ had been added. The final magnesium concentration in the medium was 64 mM./l. After 48 hr. in this medium, the haemolymph from a group of animals was pooled. The osmotic pressure had risen to become closely isotonic with the medium, but the haemolymph magnesium was not more than 1 mM./l. These observations indicate that although, following damage to the branchial epithelium, the haemolymph osmotic pressure becomes close to that of the medium, there is no great increase in indiscriminate permeability, as otherwise the haemolymph ionic composition would be the same as that of the medium. This is in keeping with the fact that the haemolymph of 'burnt' animals still looked like normal haemolymph, i.e. cherry-red with haemoglobin.

A series of observations was also made on the chemical composition of whole 'burnt' animals. The total water and chloride content were related to phosphate content as had been done with normal animals (Croghan, 1958*b*). 'Burnt' animals were kept in 25% sea water for 24 hr. The animals were then divided into two groups. One group was placed in fresh 25% sea water (= 0·83% NaCl) for a further 48 hr., and the other group was placed in 50% sea water (= 1·58% NaCl) for 48 hr. Then from each medium two groups of active animals with black or brown branchiae were taken, and the following quantities were determined: total water content, chloride content of the ash, phosphate content of the ash. The mean values of the chloride concentration in the total body water, and the chloride/phosphate and water/phosphate ratios in the two groups from 25% sea water were each taken as 100, and the mean values from the two groups of animals from 50% sea water were all expressed relative to this. The results are summarized in Table 2. It is evident that changes in haemolymph osmotic pressure are due more to changes in the total ion content of the animal than to water movements.

Table 2. *The chemical composition of whole animals 'burnt' with* $KMnO_4$

Medium	Whole animals		
Osmotic pressure	Cl in total body water	chloride / phosphate	water / phosphate
100	100	100	100
190·5	193	182·5	95

The conclusions from the preceding experiments are of interest. 'Burnt' animals lose the ability to osmo-regulate. In the more concentrated solutions the haemolymph osmotic pressure rises to become isotonic with the medium due to an increase in the total ionic content of the animal. This is not due to an increase in the indiscriminate permeability of the animal. It appears that the $KMnO_4$ treatment has destroyed the specific mechanism excreting NaCl against the concentration gradient. This, correlated with the fact that the visible damage is sharply localized to the epithelium of the first ten pairs of branchiae, and that it is only over these branchiae that the cuticle is appreciably permeable, indicates that this branchial epithelium is the site of the active transport of NaCl, which in undamaged animals maintains the haemolymph hypotonic to the more concentrated media. The same mechanism working the other way round is probably concerned with the uptake of NaCl that must occur in the dilute and hypotonic media.

Ontogeny of the regulatory mechanism

The nauplii of *Artemia* are also hypotonic to sea water (Croghan, 1958*b*), and yet in these young stages the limb buds are only just beginning to appear, and there are no branchiae. These nauplii, however, possess a large and curious structure: the neck organ, which is described and figured by Dejdar (1930). Dejdar correlated this structure with the adult branchiae. He considered that they are part of the same functional system, and that as the animal grows the branchiae form and replace the neck organ.

It is confirmed here that the silver staining of the nauplius is localized to the neck organ, and that it appears identical with that of the adult branchiae. Also, saturated $KMnO_4$ solution caused the epithelium of the neck organ to become brown and distorted and to pull away from the thin overlying cuticle, just as in adult branchiae. These reactions can, as in the branchiae, be interpreted as evidence that the cuticle over the neck organ is permeable. It is considered, therefore, that the neck organ is concerned in NaCl excretion in the nauplius and that it functions as such until the branchiae become functional. The neck organ then degenerates.

Some observations have been made on the process of replacement of the neck organ by the branchiae. The results are summarized in Table 3. The limb buds appear and develop in a sequence, the more anterior ones first. Branchiae appear and later develop the ability to stain with silver. The staining is 'all or none,'

Table 3. *The ontogeny of the branchiae*

Length of animal mm.	No. pairs limb buds	No. pairs branchiae	No. pairs Ag-stained branchiae	Neck organ Ag-stained
0·5–0·8	0	0	0	+
1·0	4–5	0	0	+
1·7	8–9	5–6	1–2	+
2·0	10	6	3	+
2·5	11	8–9	4–5	+
3·0	11	10	6	+
3·8	11	11	7	+
6 (and upwards)	11	11	10	−

and it appears that at each moult one to two more pairs of branchiae can become stained, until only the last pair is left unstained. It seems that the branchial epithelium develops initially with an impermeable cuticle. Later the mechanism for NaCl excretion develops, and then at the next or a subsequent moult a permeable cuticle is secreted. There would appear to be an interesting co-ordination of two separate epithelial functions: NaCl excretion, and the secretion of a permeable instead of an impermeable cuticle.

DISCUSSION

Koch (1934) and Krogh (1939) have regarded the uptake of silver ions by Crustacea and Insecta as directly due to an ion transport mechanism. However, the *Artemia* results reported here show that the localized uptake and precipitation of silver is a purely passive process. The results indicate only that the cuticle at these sites is permeable. The experiments with methylene blue and $KMnO_4$ solutions confirm this. It is probable that this simple explanation also holds for the many other described examples of silver staining in the Crustacea and Insecta.

It is of interest that the eleventh pair of branchiae in *Artemia*, although apparently morphologically identical with the preceding pairs, is not affected by $AgNO_3$, $KMnO_4$ or methylene blue, indicating that there is a differentiation of properties along the branchial series. A rather similar differentiation along the gill series has been observed in *Potamon* spp. by Ewer & Hattingh (1952). The structural basis of the localization of external permeability in *Artemia* has not been studied.

It seems clear that the cuticle over the first ten pairs of branchiae is the only appreciably permeable part of the external cuticle, and that the epithelium underlying this permeable cuticle is capable of actively excreting NaCl from the haemolymph into a hypertonic medium. This branchial excretory mechanism has been superimposed upon a basically fresh-water animal, and when this mechanism is destroyed the animal behaves like a fresh-water animal that has never evolved a hypotonic regulatory mechanism, and in which the upper concentration limit for survival is consequently low.

This regulatory mechanism in *Artemia* can be compared with those found in other animals that have evolved hypotonic regulation. It is very similar to that

found in the marine teleosts, in which Keys (1931) clearly demonstrated an excretion of chloride across the branchial epithelium. In both types the epithelium on the most permeable part of the external surface excretes NaCl against the concentration gradient. It is probable also that a similar mechanism operates in the marine palaemonids investigated by Panikkar (1941*b*).

These branchial mechanisms can be contrasted with that found in *Aedes detritus* larvae (Beadle, 1939; Ramsay, 1950). Here the same problem of excreting NaCl against a concentration gradient has been solved in a different way. The Malpighian tubules and rectal epithelium form an organ system that ultimately produces a concentrated fluid. In very dilute media, however, this rectal fluid can become hypotonic to the haemolymph. This may perhaps explain the prolonged survival of this animal in distilled water, which is a sharp contrast to *Artemia*.

The branchial excretory mechanism is not, however, the complete explanation of osmotic regulation in *Artemia*, and in a subsequent paper (Croghan, 1958*c*), the physiology of the gut will be considered in relation to osmotic regulation.

SUMMARY

1. The uptake of silver ions by *Artemia* has been investigated. The staining is localized to the first ten pairs of branchiae. There is no staining of the eleventh pair or of any other part of the animal. The uptake of silver is due to a purely passive precipitation of AgCl within the thickness of the branchial cuticle.

2. The effects of $KMnO_4$ and methylene-blue solutions have also been studied. Their effect is localized to the epithelium under the cuticle of the first ten pairs of branchiae.

3. It is concluded that all these staining reactions demonstrate that the cuticle over the first ten pairs of branchiae is the only part of the external cuticle that is appreciably permeable.

4. Animals whose branchial epithelium has been damaged by a brief exposure to saturated $KMnO_4$ solution have lost the ability to osmo-regulate. They are closely isotonic with their medium, and the range of external concentration tolerated is much restricted.

5. This isotonicity is not due simply to increased permeability, but is due to specific destruction of the mechanism normally excreting NaCl in hypertonic media.

6. Correlation of the physiological effects of $KMnO_4$ treatment with the sharp localization of damage, and the evidence for localized permeability indicates that the epithelium of the first ten pairs of branchiae is the site of active NaCl excretion in hypertonic media, and probably of active uptake from hypotonic media.

7. The ontogeny of this mechanism is traced. In nauplii the dorsal organ is apparently concerned in NaCl excretion. When the branchiae develop the dorsal organ degenerates.

I wish to thank Dr J. A. Ramsay, F.R.S., for his interest and advice. I also wish to thank the Department of Scientific and Industrial Research for a maintenance grant.

REFERENCES

BEADLE, L. C. (1939). Regulation of the haemolymph in the saline water mosquito larva *Aedes detritus* Edw. *J. Exp. Biol.* **16**, 346.

CROGHAN, P. C. (1958*a*). The survival of *Artemia salina* (L.) in various media. *J. Exp. Biol.* **35**, 213.

CROGHAN, P. C. (1958*b*). The osmotic and ionic regulation of *Artemia salina* (L.). *J. Exp. Biol.* **35**, 219.

CROGHAN, P. C. (1958*c*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut. *J. Exp. Biol.* **35**, 243.

DEJDAR, E. (1930). Die Korrelationen zwischen Kiemensäckchen und Nackenschild bei Phyllopoden. *Z. wiss. Zool.* **136**, 422.

EWER, D. W. & HATTINGH, I. (1952). Absorption of silver on the gills of a freshwater crab. *Nature, Lond.*, **169**, 460.

KEYS, A. B. (1931). Chloride and water secretion and absorption by the gills of the eel. *Z. vergl. Physiol.* **15**, 364.

KOCH, H. J. (1934). Essai d'interprétation de la soi-disant 'réduction vitale' de sels d'argent par certains organes d'arthropodes. *Ann. Soc. Sci. méd. nat. Brux.* B, **54**, 346.

KROGH, A. (1939). *Osmotic Regulation in Aquatic Animals.* Cambridge.

PANIKKAR, N. K. (1941*a*). Osmotic behaviour of the fairy shrimp *Chirocephalus diaphanus* Prévost. *J. Exp. Biol.* **18**, 110.

PANIKKAR, N. K. (1941*b*). Osmoregulation in some palaemonid prawns. *J. Mar. Biol. Ass.* **25**, 317.

RAMSAY, J. A. (1950). Osmotic regulation in mosquito larvae. *J. Exp. Biol.* **27**, 145.

THE MECHANISM OF OSMOTIC REGULATION IN *ARTEMIA SALINA* (L.): THE PHYSIOLOGY OF THE GUT

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INTRODUCTION

In preceding papers various aspects of the osmotic regulation of *Artemia* have been described (Croghan, 1958*b*, *c*). The importance of the branchial mechanism excreting NaCl has been discussed (Croghan, 1958*c*), but it is clear that in order to maintain a haemolymph steady state in hypertonic media a mechanism for the active uptake of water must also be present.

The gut epithelium appears to be the most permeable part of *Artemia*, and it seems clear that in hypertonic media it is across this epithelium that there will be the greatest tendency for NaCl to enter and for water to leave the haemolymph (Croghan, 1958*b*). In this paper further studies on the physiology of the gut in relation to osmotic regulation are described. The gut of *Artemia* is a simple straight tube formed of a single layer of epithelial cells, and has in the head region a pair of small globular diverticula. A thin peritrophic membrane is present in the gut tube.

MATERIAL AND METHODS

Adult *Artemia* as described previously (Croghan, 1958*a*) were used.

Samples of gut fluid were obtained as follows. First, a haemolymph sample was taken, if required, as described previously (Croghan, 1958*b*). Then, still working under liquid paraffin, the animal was dissected away from the gut tube, and any adherent haemolymph was sucked away with a fine pipette. Then the gut wall was punctured and the gut contents sucked up into another pipette. Gentle squeezing of the gut with a needle facilitated collection. Although sufficient fluid for a freezing-point determination could be obtained from a single animal, in most cases samples from ten to twenty animals were pooled for chemical analyses. The gut fluids were sucked into a fine tube and centrifuged. The clear yellow-green supernatant appeared quite different from the cherry-red haemolymph, and showed no trace of haemoglobin absorption bands. In many cases $BaSO_4$ was suspended in the medium some time before the animals were sampled. This was swallowed and cleared the gut of organic and cellular debris. It also had the advantage of providing extra bulk for collecting the gut fluid and also centrifuged down cleanly.

Freezing-point determinations and chemical analyses of the haemolymph and gut

fluids were carried out by methods described previously (Croghan, 1958*b*), except that the potassium analyses on the gut fluids were carried out by the method of Ramsay, Brown & Falloon (1953).

RESULTS

Swallowing behaviour

Active animals continuously filter-feed from the medium, and fine particles of charcoal or $BaSO_4$ added as a suspension to the medium are rapidly taken into the gut. On changing the animal from a suspension of say $BaSO_4$ to charcoal, a sharp colour interface in the gut contents rapidly passes back along the intestinal tube.

The animal can also be shown to be actively swallowing its medium. Phenol red was dissolved in the medium and the solution filtered. Animals were placed in these media. Within a few hours the entire gut lumen was bright red, indicating that the medium had been swallowed. This swallowing has been observed in all saline media, hyper-, iso- and hypotonic. The dye was confined to the gut lumen and did not enter any other part of the animal. It appeared to be more concentrated in the gut than in the medium.

In animals ligatured at the anus, rapid oral swallowing still occurs, and the gut lumen rapidly becomes bright red. Animals ligatured at the neck show a somewhat more variable behaviour, but in most there is evidence of anal swallowing, the rectum first becoming bright red and the dye then spreading slowly forwards.

Samples of gut fluid were obtained from animals kept in media containing dissolved phenol red. The results indicated that the dye was in solution in the gut contents and that it was more concentrated than in the medium being swallowed. Some animals were placed in a dilute filtered solution of phenol red in sea water. After 24 hr. the gut contents of about fifteen large animals were pooled and centrifuged to remove food particles. The supernatant, a tiny drop of a very dark red fluid, was pipetted into 1 ml. of dilute ammonia solution. Standards were prepared by using the same pipette and adding different volumes of the coloured medium to 1 ml. volumes of ammonia solution. The final solutions were compared in a Spekker absorptiometer using 2 cm. light path microcells and blue filters. No dye could be detected in the haemolymph, but in the gut lumen the phenol red was about seven times more concentrated than in the medium. These results demonstrate that *Artemia* rapidly swallows the medium and takes up water from it, thus concentrating the dye.

Some animals that had swallowed filtered phenol red sea water for a few hours were placed in filtered uncoloured sea water. Dye was retained in the gut lumen for prolonged periods. Many still retained appreciable amounts even after 72 hr. This confirms that the medium is swallowed at both ends of the gut and that water is taken up from the lumen. On transferring to the uncoloured medium swallowing continues, and thus the dye already in the lumen persists for long periods.

Composition of the gut fluids

The animals were acclimatized in clean-filtered media covering a wide concentration range, and then usually 2–3 days before the animals were sampled small amounts of $BaSO_4$ powder were added. Samples of the haemolymph, gut fluid and medium were obtained. In order to obtain sufficient volumes the gut fluids of a number of animals were usually pooled. The haemolymph samples from the same animals were also pooled for comparison with the gut fluid.

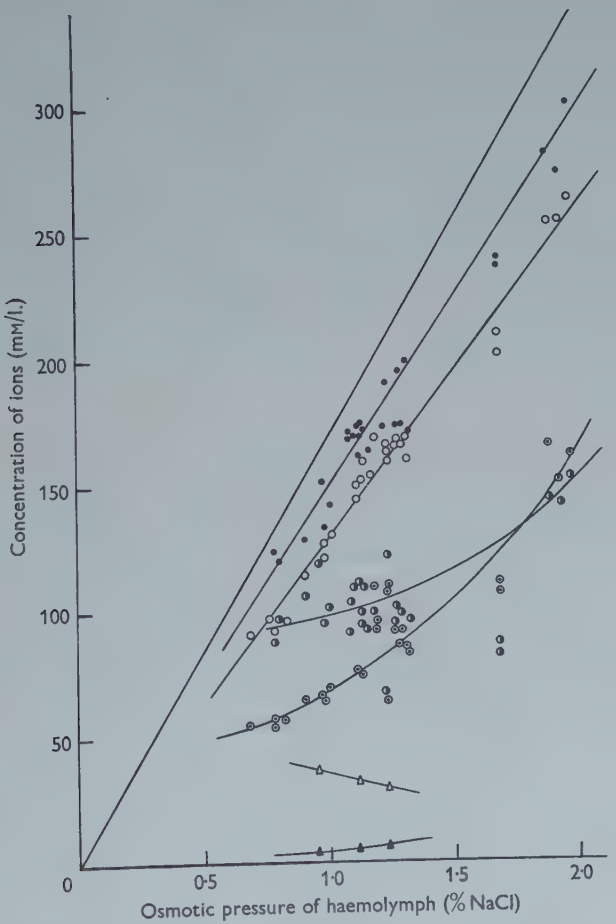

Fig. 1. The chemical composition of the gut fluid in comparison with the haemolymph from the same group of animals. Sodium concentration in haemolymph, ●; in gut fluid, ◑. Chloride concentration in haemolymph, ○; in gut fluid, ⊙. Potassium concentration in haemolymph, ▲; in gut fluid, △.

For convenience these results on the osmotic pressure of the gut fluids, haemolymph and medium have been included as part of Figs. 1 and 2 in an earlier paper (Croghan, 1958*b*). The osmotic pressure of the gut fluids is always greater than that

of the haemolymph, but in the more concentrated media the osmotic pressure of the gut fluids is still very much less than that of the medium, although, as we have seen, the animal is continuously swallowing that medium and taking up water from it. This demonstrates that large amounts of NaCl must be entering the haemolymph across the gut epithelium.

In addition to osmotic pressure determinations, chemical analyses have also been made on many of these samples. The ionic composition of the gut fluid, and of the haemolymph from the same group of animals, is plotted as a function of the haemolymph osmotic pressure (Fig. 1). Although the osmotic pressure of the gut fluid is always greater than the corresponding haemolymph, the concentrations of sodium and chloride in the gut fluid are considerably less than in the haemolymph. This confirms that large amounts of NaCl are entering the haemolymph across the gut epithelium.

Mention should be made of the occurrence of crystals in the gut lumen of *Artemia*. Kuenen (1939) gave a bibliography of this subject, but failed to produce crystals experimentally. Plattner (1955) observed crystals in the gut lumen when the animals were living in the more concentrated media. I have found that crystals appeared when the animals were living in high salinities (from *c.* 600% up to saturated sea water). The gut lumen contained numerous crystals, which were sometimes very big and clearly must have crystallized *in situ*. These crystals were strongly birefringent.

DISCUSSION

It is to be expected that *Artemia* would swallow some of its medium as a result of filter-feeding activity, but it has been shown also that the animal continues to swallow its medium even when no particles are present. This is a result of oral and anal swallowing, such as has been described in many Crustacea, including also *Artemia*, by Fox (1952). It is difficult to say whether this swallowing in *Artemia* is an essential part of the normal feeding mechanism continuing in the absence of food particles or a completely independent physiological process.

It has been shown that *Artemia* takes up water into the haemolymph from the swallowed medium, even when the medium is markedly hypertonic to the blood. There is thus in the gut epithelium a mechanism for the active uptake of water.

Although *Artemia* swallows a hypertonic medium and takes up water from it, the osmotic pressure of the gut fluid is well below that of the medium. This shows that large amounts of NaCl must be entering the haemolymph across the gut epithelium. NaCl would initially tend to diffuse in from the swallowed fluid, but the chemical analyses show that the concentration of both the sodium and chloride ion in the gut fluids is well below that in the haemolymph. An active uptake of NaCl across the gut epithelium must therefore exist.

An attempt can be made to interpret the physiology of the gut in hypertonic media. A permeable animal in a hypertonic medium would tend constantly to be dehydrated. But in *Artemia* the permeability of the external cuticle is low and appreciable permeability has become restricted to the gut epithelium. In order to

prevent the ingestion of concentrated medium from dehydrating the animal it would be necessary for the animal to take up actively sufficient water at least to balance that which is tending to move passively from the haemolymph into the gut lumen. But epithelia in general, apart possibly from parts of the mammalian kidney tubule, do not seem to have evolved or developed any mechanism that can directly transport water against large osmotic gradients. The active uptake of NaCl from the gut fluids can be interpreted as an indirect way of solving this physiological problem. The active uptake of NaCl lowers the osmotic pressure of the gut contents to a level that the active mechanisms for water uptake can overcome. The NaCl entering the haemolymph across the gut epithelium would be excreted by the branchiae. Judging by the high concentration reached by swallowed dye solutions in the gut, the system works with an appreciable net gain of water for the animal. Some of this excess water would balance the slight osmotic water losses that would occur through the branchial and the rest of the external cuticle. The rest must be excreted by the maxillary glands. Unfortunately, owing to their small size, it was impossible to study the maxillary glands in relation to osmotic regulation.

A large part of the gut fluid osmotic pressure cannot be accounted for by sodium, potassium or chloride ions. It is possible that much of this deficit is made up by divalent ions (magnesium, calcium, sulphate) from the swallowed medium. These divalent ions would be less likely to be taken up across the gut epithelium than the univalent ions and so would rise in concentration as water was taken up from the gut fluids. Unfortunately, it was not possible to determine the concentration of these ions in the very small quantities of gut fluids available. It is also likely that organic substances contribute appreciably to the gut fluid osmotic pressure. The rise in concentration of some substances in the gut fluid due to water absorption could also explain the appearance of crystals in the gut lumen in animals in very concentrated media. Under these conditions it would be quite likely for say $CaSO_4$ to crystallize in the gut. However, these gut crystals were not further studied.

The results reported in this paper are in sharp contradiction to some previously published work on the gut of *Artemia*. Boone & Baas Becking (1931) and Kuenen (1939) claimed that *Artemia* can effectively seal its gut off from the medium for prolonged periods. The results obtained here, however, show that, although there may be slight individual variation, swallowing is continuous in all active animals whatever the salinity of the medium. If there is any cessation of swallowing on transfer to a new medium, it can only be a temporary shock reaction and can be of no significance in the permanent adaptation to the new medium. Kuenen claimed that not only was the gut isolated, but that it also acted as a storage chamber for water. Much of his evidence is dubious and difficult to accept. For example, he calculates expected volume changes on transferring *Artemia* to a medium of different concentration on the assumption that changes in blood concentration (as measured by refractive index) represent water movements into or out of the haemolymph. He ignores salt movements, yet as has been shown (Croghan, 1957*b*), these are responsible for most of the changes in haemolymph concentration. Kuenen's conclusions are not, I believe, justified.

A permeable animal living in a medium that has a different concentration from the haemolymph must possess mechanisms that can control both the NaCl and water balances. The combination of the branchial mechanism (maintaining NaCl balance) and the gut mechanism (maintaining water balance) are considered to be the mechanisms that superimposed upon a fresh-water type of organization have enabled *Artemia* to colonize highly saline waters.

These osmo-regulatory mechanisms in *Artemia* can be compared with those found in other types that have evolved hypotonic regulation. The *Artemia* mechanisms appear very similar to those found in the marine teleosts. The similarity of the branchial mechanisms excreting NaCl has already been stressed (Croghan, 1958*c*). Further, there seems also to be a close similarity in the physiology of the gut. Smith (1930, 1932) found that the marine teleost also swallows its medium and takes up water from it. The osmotic pressure of the gut fluids falls to become approximately isotonic with the blood due to the uptake of NaCl. The uptake of NaCl is also apparently active, since the concentration of these ions falls below that in the blood. He has also shown that divalent ions become concentrated in the gut. An interesting but unexplained difference, however, is that in *Artemia* the gut potassium concentration is high, whereas in the marine teleosts it is very low. Further, no data are available concerning the concentration of the divalent ions in the *Artemia* gut fluids. It seems clear, however, that in both the marine teleosts and in *Artemia* the gut is functioning in a closely identical manner as an organ actively taking up water.

The evolution of osmotic regulation in these two quite distinct groups appears to show a remarkable convergence. In both types there is a branchial excretion of NaCl, and in both the physiology of the gut in relation to water balance regulation appears similar.

There are less data available concerning the gut physiology of *Aedes detritus* larvae. But the results of Beadle (1939) and Ramsay (1950) show that, as in *Artemia*, the permeability of the animal is restricted to the gut epithelium, that the larva swallows the medium, and that the osmotic pressure of the ingested fluid falls in the midgut. It seems probable that this region of the *Aedes* gut is functioning in relation to water balance in a way similar to the gut of *Artemia*. There is almost nothing known about the gut physiology of the marine palaemonids, but here also it is quite likely that the gut is important in maintaining water balance.

SUMMARY

1. *Artemia* is continuously swallowing its medium, whether it is hyper-, iso-, or hypotonic to the haemolymph, and taking up water from the gut lumen.

2. The osmotic pressure of the gut fluids is appreciably greater than that of the haemolymph, but in the more concentrated media is considerably below that of the medium. This indicates that considerable amounts of NaCl must be passing across the gut epithelium into the haemolymph.

3. The concentration of both sodium and chloride ions in the gut fluids is always

less than that in the haemolymph, indicating that there must be an active uptake of NaCl across the gut epithelium.

4. It is considered that the gut of *Artemia* has become adapted as a mechanism for the active uptake of water, controlling water balance and preventing dehydration in hypertonic media.

5. The adaptations for maintaining the NaCl and the water balances in *Artemia* are compared to those found in the marine teleosts, and are shown to be extremely similar.

Again, I wish to thank Dr J. A. Ramsay, F.R.S., for his interest and advice, and also for carrying out the potassium analyses on the gut fluids. I also wish to thank the Department of Scientific and Industrial Research for a maintenance grant.

REFERENCES

Beadle, L. C. (1939). Regulation of the haemolymph in the saline water mosquito larva *Aedes detritus* Edw. *J. Exp. Biol.* **16**, 346.

Boone, E. & Baas Becking, L. G. M. (1931). Salt effects on eggs and nauplii of *Artemia salina* L. *J. gen. Physiol.* **14**, 753.

Croghan, P. C. (1958*a*). The survival of *Artemia salina* (L.) in various media. *J. Exp. Biol.* **35**, 213.

Croghan, P. C. (1958*b*). The osmotic and ionic regulation of *Artemia salina* (L.). *J. Exp. Biol.* **35**, 219.

Croghan, P. C. (1958*c*). The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. *J. Exp. Biol.* **35**, 234.

Fox, H. M. (1952). Anal and oral intake of water by Crustacea. *J. Exp. Biol.* **29**, 583.

Kuenen, D. J. (1939). Systematical and physiological notes on the brine shrimp, *Artemia*. *Arch. néer. Zool.* **3**, 365.

Plattner, F. (1955). Der osmotische Druck von *Artemia salina*. *Pflüg. Arch. ges. Physiol.* **261**, 172.

Ramsay, J. A. (1950). Osmotic regulation in mosquito larvae. *J. Exp. Biol.* **27**, 145.

Ramsay, J. A., Brown, R. H. J. & Falloon, S. W. H. W. (1953). Simultaneous determination of sodium and potassium in small volumes of fluid by flame photometry. *J. Exp. Biol.* **30**, 1.

Smith, H. W. (1930). The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.* **93**, 480.

Smith, H. W. (1932). Water regulation and its evolution in fishes. *Quart. Rev. Biol.* **7**, 1.

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